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Unravelling the Causes of Inner Ear Disease

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Abbreviations

µL	Microliter
µg	Microgram
ºC	Celsius Degrees
AAO-HNS	American Academy of Otolaryngology - Head and Neck Surgery
AMP	Adenosine Monophosphate
ARHL	Age-Related Hearing Loss
ATP	Adenosine Triphosphate
ATP1A2	ATPase Na^+/K^+ Transporting Subunit Alpha 2
bp	Base Pairs
dB	Decibel
CACNA1A	Calcium Voltage-Gated Channel Subunit Alpha1 A
cAMP	Cyclic Adenosine Monophosphate
CANVAS	Cerebellar Ataxia, Neuropathy, Vestibular Areflexia Syndrome
CCR7	C-C Motif Chemokine Receptor 7
CD	Cluster of Differentiation
CD40LG	Cluster of Differentiation 40 Ligand
CI	Confidence Interval
cDNA	Complementary Deoxyribonucleic Acid
CNV	Central Nervous System
CO_2	Carbon Dioxide
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DRB1	DR-1 Beta Chain
dsDNase	Double Strand Specific DNase
E2F1	E2F Transcription Factor 1
ER	Endoplasmic Reticulum
ES	Endolymphatic Sac
FHM	Familial Hemiplegic Migraine
FMD	Familial Meniere's Disease
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
gDNA	Genomic Deoxyribonucleic Acid
GFI1	Growth Factor Independent Factor 1
GRM7	Glutamate Metabotrophic Receptor 7
HGF	Hepatic Growth Factor
HIF1A	Hypoxia-inducible Factor 1α
HL	Hearing Loss
HRH1	Histamine Receptor H1
HRH3	Histamine Receptor H3
HVS1	Hypervariable Segment 1
Hz	Hertz

I	Intermediate
iCOS	Inducible T-cell Co-stimulator
iCOSL	Inducible T-cell Co-stimulator Ligand
IFN	Interferon
IFNA2	Interferon type 2
IFNG	Interferon γ
IgE	Immunoglobulin E
IKKe	IkB kinase ε
IL	Interleukin
IL-1β	Interleukin 1 β
IL-1RN	Interleukin 1 Receptor Antagonist (gene)
IL-1RA	Interleukin 1 Receptor Antagonist (protein)
IL-6	Interleukin 6
IRF3	Interferon Regulatory Factor 3
K^+	Potassium Ion
kDa	Kilodalton
LYP	Lymphoid Protein Tyrosine Phosphatase
MAPK	Mitogen-Activated Protein Kinase 1
MD	Meniere's Disease
MHC	Major Histocompatibility Complex
mL	Millilitre
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
mtDNA	Mitochondrial Deoxyribonucleic Acid
mTOR	Mechanistic Target Of Rapamycin
mTORC2	Mechanistic Target Of Rapamycin Complex 2
MYC	MYC Proto-Oncogene, BHLH Transcription Factor
MyD88	Myeloid Differentiation Primary Response 88
Na^+	Sodium Ion
NAT2	N-acetyltransferase 2
NFkB	Nuclear Factor-κB
NMDA	N-methyl-D-aspartate
NSHL	Non-syndromic Hearing Loss
OR	Odds Ratio
PAMP	Ppathogen-associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pg	Picogram
PI3K	Phosphoinositide 3-kinases
PKA	Protein Kinase A

PRL	Prolactin
PRR	Pattern Recognition Receptors
PTGER4	Prostaglandin E receptor subtypes EP4
qPCR	Quantitative Polymerase Chain Reaction
R	Rapid
RICTOR	RPTOR Independent Companion Of MTOR Complex 2
RIP1	Receptor Interacting Serine/Threonine 1
RNA	Ribonucleic Acid
rpm	Rotations per minute
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
S	Slow
SCN1A	Sodium Voltage-Gated Channel Alpha Subunit 1
SD	Standard Deviation
SE	Standard Error
SNHL	Sensorineural Hearing Loss
SNP	Single Nucleotide Polymorphism
STAT1	Signal Transducer And Activator Of Transcription 1
TAK1	Transforming Growth Factor Beta-activated Kinase 1
TBE	Tris-Borate- Ethylenediaminetetraacetic Acid
TBK1	TANK Binding Kinase 1
TCR	T-cell Receptor
Th	T Helper Cell
TLR	Toll-like Receptor
TNFα	Tumour Necrosis Factor α
TRAM	Translocation Associated Membrane Protein
T_{REG}	T Regulatory Cell
TRIF	TIR Domain-containing Adaptor Protein Inducing Interferon Beta
TRIM24	Tripartite Motif Containing 24
tRNA	Transfer Ribonucleic Acid
U	Enzyme Units
UPR	Unfolded Protein Response
VD	Vestibular Disorders
VEGF	Vascular Endothelial Growth Factor
VM	Vestibular Migraine
WHO	World Health Organization
XBP1	X-box-binding Protein 1

Resumo

Introdução: O ouvido interno é o órgão sensorial responsável pela manutenção do equilíbrio e pela capacidade auditiva. A surdez é a deficiência neuro-sensorial mais comum no Ser Humano, podendo ter uma origem genética e/ou ambiental. A presbiacusia, ou surdez associada à idade, é um tipo de surdez neuro-sensorial progressivo e bilateral, que resulta do efeito cumulativo de fatores intrínsecos e extrínsecos, levando à degeneração da cóclea e das vias centrais auditivas. Estudos prévios indicam uma predisposição genética para o desenvolvimento de presbiacusia. A surdez neuro-sensorial pode ocorrer associada a outros sintomas e ser intermitente em algumas manifestações como na doença de Meniere. A doença de Meniere é uma doença multifatorial, caraterizada por ataques de vertigens espontâneos, associado a surdez neuro-sensorial unilateral intermitente, acufenos e uma sensação de preenchimento do ouvido ("aura"). Os pacientes com doença de Meniere podem também desenvolver surdez neuro-sensorial bilateral, enxaquecas e doenças autoimunes sistémicas. Apesar da doença de Meniere ser considerada multifatorial, existem três hipóteses mais aceites para a sua etiologia - genética, autoimune ou alérgica – existindo evidências científicas para todas elas. A prevalência de alergias diagnosticadas em pacientes de doença de Meniere é três vezes superior à população geral. Nas doenças do ouvido há também a considerar as doenças vestibulares, que levam à perda transiente ou permanente da função vestibular. A Enxaqueca Vestibular é caraterizada pela ocorrência de episódios de sintomas vestibulares e uma história de enxaqueca, com uma associação temporal em pelo menos 50% dos ataques. A Enxaqueca Vestibular é mais comum em pacientes com doença de Meniere do que na população geral, podendo por vezes serem indistinguíveis baseado apenas em historial médico e sintomas, podendo também apresentar-se como uma comorbidade. Porém, a relação patofisiológica entre Enxaqueca Vestibular e doença de Meniere é desconhecida.

Objetivos: Caracterização genética de pacientes de presbiacusia a nível dos genes *GRM7* e *NAT2* e haplogrupos de mtDNA; comparação da resposta imune induzida por fungos entre pacientes de doença de Meniere e Enxaqueca Vestibular.

Materiais e Métodos: Estudo e análise das caraterísticas demográficas e clinicas dos indivíduos da amostra de presbiacusia (n=483, oriunda da população portuguesa), doença de Meniere (n=115, oriundas da população espanhola) e Enxaqueca Vestibular (n=73, oriundas da população espanhola); sequenciação da região *HVS1* do DNA mitocondrial e determinação de haplogrupos utilizando o mtDNA Haplogroup Analysis *software*; genotipagem por real-time PCR dos genes *GRM7* (rs11928865) e *NAT2* (rs1799930, rs1799931, rs1801280, rs1208, rs1041983, rs1799929 e rs1801279). Determinação do fenótipo de acetilação do gene *NAT2* utilizando NAT2PRED *software*. Determinação do nível de expressão de citocinas (IL-1β, IL-1RN, IL-6 e TNFα) em amostras de indivíduos com Enxaqueca Vestibular por PCR quantitativo e quantificação do nível de liberação extracelular das mesmas utilizando um Multiplex Bead-Based Kit; determinação da expressão génica por *microarray* de Enxaqueca Vestibular e Doença de Meniere; determinação das vias canónicas, identificação de reguladores e predição do estado (ativo/inibido) utilizando IPA® *software*.

Resultados e Discussão: Os nossos resultados demonstram que na amostra de pacientes com presbiacusia há diferenças significativas entre a idade e o nível de perda auditiva, os homens têm uma melhor audição nas baixas frequências e as mulheres nas altas frequências evidenciando um efeito de inversão. Distintas comorbidades foram associadas a presbiacusia, nomeadamente: níveis de colesterol elevado, hipertensão, doença cardiovascular e hábitos tabágicos. O genótipo T/T do SNP rs11928865 do gene *GRM7* confere uma maior perda auditiva a 8000 Hz. O haplótipo NAT2*4/NAT2*6A está associado a uma maior perda auditiva a 250 Hz. O fenótipo de acetilação lenta, no gene *NAT2*, tem uma maior perda auditiva, porém não existem diferenças significativas entre este fenótipo e os restantes. A origem dos haplogrupos mais detetados na nossa amostra é europeia, sendo o haplogrupo mais comum

o haplogrupo H, porém também foram identificados haplogrupos asiáticos e um haplogrupo africano. Apesar de não terem sido encontradas diferenças significativas, o haplogrupo com maior perda auditiva é o haplogrupo X. Os resultados de qPCR revelam que os pacientes com Enxaqueca Vestibular comparativamente a controlos saudáveis, quando estimulados com *Aspergillus* têm uma maior expressão de IL-1, TNFα e IL-6, porém apenas a diferença em IL-6 é estatisticamente significativa. No entanto, não se vê uma diferença na expressão de citocinas após estimulação com *Penicillium*. Os níveis basais de IL-1RA são mais elevados em Enxaqueca Vestibular do que nos controlos. Os níveis de IL-6 e TNFα são mais elevados em Enxaqueca Vestibular do que em controlos após estimulação com *Aspergillus* e *Penicillium*. Não existem diferenças significativas entre os níveis basais de Enxaqueca Vestibular e doença de Meniere com baixos níveis de IL-1β, porém existem diferenças significativas entre os níveis de IL-1RA de Enxaqueca Vestibular e doença de Meniere com baixos níveis de TNFα e entre os níveis de IL-1β, IL-1RA, IL-6 e TNFα de EV e doença de Meniere com elevados níveis de IL-1β e TNFα. Após estimulação, verifica-se uma diferença nos níveis de IL-1RA e IL-6 entre Enxaqueca Vestibular e doença de Meniere com baixos níveis de IL-1β e TNFα e uma diferença entre os níveis de IL-1RA entre Enxaqueca Vestibular e doença de Meniere com elevados níveis de IL-1β e TNFα. Não existem diferenças significativas entre os perfis de expressão génica entre pacientes de Enxaqueca Vestibular e controlos saudáveis e entre pacientes de Enxaqueca Vestibular e pacientes de doença de Meniere com baixos níveis de citocinas. Porém, existem diferenças entre pacientes com Enxaqueca Vestibular e pacientes com Doença de Meniere com elevados níveis de citocinas, que revelam o envolvimento da maturação de linfócitos B, produção de citocinas e a maturação de células dendríticas. A comparação da resposta a *Aspergillus* e *Penicillium* entre pacientes com Enxaqueca Vestibular e controlos saudáveis revelou diferenças no envolvimento da produção de citocinas, inflamação e da sinalização para a morte celular programada. A comparação da resposta a estimulação com *Aspergillus* e *Penicillium* entre pacientes com Enxaqueca Vestibular e Doença de Meniere revelou o envolvimento de reguladores que atuam na diferenciação, ativação e regulação de linfócitos T, no desenvolvimento de linfócitos B, na maturação de células dendríticas e na alergia.

Conclusão: As doenças do ouvido interno podem ter várias causas e comorbidades associadas. Na nossa amostra de presbiacusia, os haplogrupos de mtDNA não mostram qualquer efeito na perda auditiva. Não se pode excluir o efeito genético de *GRM7*, *NAT2* e haplogrupos do mtDNA na presbiacusia, no entanto na nossa amostra os fatores ambientais poderão ter um maior efeito para o desenvolvimento de presbiacusia do que os fatores genéticos analisados, ainda que seja de considerar o possível efeito de outros fatores genéticos nomeadamente os associados às alterações do colesterol. Os pacientes de Enxaqueca Vestibular podem ser distinguidos de controlos saudáveis e de pacientes de Doença de Meniere a partir dos níveis basais de IL-1RA. Os resultados do *microarray* suportam os resultados obtidos através na quantificação dos níveis extracelulares de IL-1β, IL-1RA, IL-6 e TNFα e sugerem uma resposta alérgica nos pacientes de Enxaqueca Vestibular.

Palavras-chave: Ouvido interno; Presbiacusia; Doença de Meniere; Enxaqueca Vestibular

Abstract

Introduction: The inner ear is a sensory organ responsible for the maintenance of balance and equilibrium and hearing. Age-related hearing loss (ARHL) results from the cumulative effect of intrinsic and extrinsic factors that lead to degenerative changes related to ageing of the cochlea and central auditory pathways. Meniere's Disease (MD) is a multifactorial disorder characterized by vertigo attacks usually associated to unilateral sensorineural hearing loss (SNHL), tinnitus and aural fullness. MD patients have higher prevalence of diagnosed allergy than the general population. Vestibular Migraine (VM) is defined by occurrence of episodic vestibular symptoms and a history of migraine, however it has high symptom overlapping with other vestibular disorders, such as MD.

Objectives: Genetically characterize ARHL patients at *GRM7*, *NAT2* and mtDNA haplogroups and compare the mold-induced immune response in VM and MD patients.

Materials and Methods: Demographic and clinical characterization of ARHL, VM and MD cohorts. *HVS1* region sequencing and mtDNA haplogroup determination; *NAT2* and *GRM7* analysis by Real time PCR; cytokine expression level determination by qPCR and extracellular cytokine quantification with Multiplex Bead-Based Kit and gene expression determining by microarray.

Results: Cholesterol, hypertension, smoking and cardiovascular disease were identified as ARHL comorbidities. GRM7 genotypes have significant differences for hearing loss (HL) at 8000 Hz. NAT2*4/NAT2*6A is associated to worst listening at 250 Hz. The haplogroups identified in our sample are mostly European. Haplogroup X has highest HL (not statistically significant). Allergenic extracts induce a proinflammatory immune response involving TNF-α and IL-6. Stimulation leads to the involvement of upstream regulators that take part in differentiation, activation and regulation of T-cells, B-cell development, dendritic cell maturation and allergy.

Conclusion: Other variables may prevail over the genetic factor in the Portuguese population of ARHL. Basal levels of IL1-RA may allow the distinction of VM and MD. The microarray data is supportive of the findings in the extracellular cytokine measurements and suggests an allergic response in VM.

Key-words: Inner ear; Age-related Hearing Loss, Meniere's Disease; Vestibular Migraine

1. Introduction

1.1. The Inner Ear

The inner ear is a sensory organ that consists of an osseous labyrinth of spaces in the temporal bone. Within it there is a fluid-filled membranous labyrinth, connected to the osseous labyrinth in various places. The endolymph fills the membranous labyrinth and the perilymph surrounds the perimembranous space. The perimembranous space is directly connected with the subarachnoid space, therefore the perilymph has a similar composition to cerebrospinal fluid, with low [K^+] and high [Na^+]. Oppositely, the membranous labyrinth is a closed system within the endolymphatic sac filled with endolymph, which has high [K^+] and low [Na^+] [1,2].

The endolymphatic sac (ES) (figure 1.1) is part of the membranous labyrinth and it is believed to have a role in fluid and ion transport, maintaining the homeostasis of the endolymph and perilymph within the inner ear, however there is no conclusive data [3].

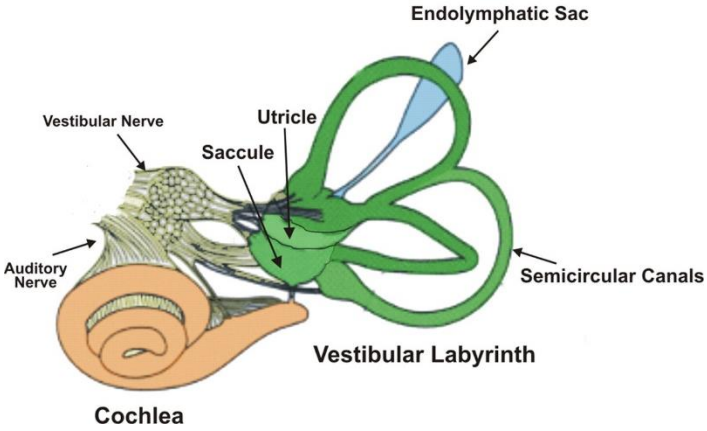

Figure 1.1 - Diagram of the inner ear. Adapted from Lang et al (3).

The osseous labyrinth is composed by the vestibule, the semicircular canals and the cochlea (figure 1). The vestibule contains the utricle and the saccule, two components of the membranous labyrinth, which are connected by the endolymphatic duct. The macula is a specialised area of the sensory receptor cells, located at the walls of the utricle and the saccule, from which axons pass into the vestibular nerve as part of sensory inputs to maintain equilibrium. [1].

The semicircular canals contain sensory receptors with axons converging on the vestibular nerve. Alongside with the macula, these receptors help maintain balance and equilibrium [1,2].

The cochlea is a conical helix in the temporal bone, its lumen is continuous with that of the vestibule. The membranous canal of the cochlea arises from the saccule and spirals towards the apex of the osseous space and can be divided into three compartments. The *scala media* (middle compartment) contains endolymph and the *scala vestibuli* (upper compartment) and *scala tympani* (lower compartment) contain perilymph. The sound sensory receptors are located at the Organ of Corti [1,2].

The organ of Corti, which lies in the *scala media* and rests on the basilar membrane, is a sensory epithelium structure containing receptor cells that allow hearing to happen. The organ of Corti vibrates in response to fluid movements and propagates this mechanical energy to the entire structure. Sound provokes a general wave within the cochlea, however the basilar membrane has different restricted response movements according to the sound frequency, as it is thinner at the base and thicker at the apex. The highest frequencies are sensed towards the base of the cochlea and gradually lower frequencies are sensed along the spiral towards the apex. For each frequency a specific point of the basilar membrane and organ of Corti vibrate and activate the appropriate hair cells to initiate afferent sensory impulses and consequentially send it to the auditory cortex of the brain. This frequency-specific property of the organ of Corti is maintained in the auditory pathway from the hair cell synapse to the auditory cortex and it is known as tonotopia. The inner hair cell synapses mainly transmit information about the acoustic environment, while the outer hair cells are involved in sound amplification. Auditory acuity can be enhanced by modulation of receptor activity through efferent suppressor pathways. This

happens due to the integrations of the sensory input from the cochlea in the brain stem and auditory cortex [1,4].

1.1.1. Inner Ear Disease

Inner ear disease classically involves the membranous labyrinth and is characterized by the triad of sensorineural hearing loss (SNHL), tinnitus and vertigo. This may be caused by alterations in the inner ear hair cells, supporting cells or an aberrant inner ear homeostasis, altering the composition of endolymph and perilymph, affecting the integrity and functionality of the hair cells. Also, altered afferent and efferent auditory pathways may accompany or cause inner ear symptoms [5].

1.2. Auditory Disorders

Hearing loss (HL) is the most common neurosensory disorder in humans, affecting 360 million people worldwide [6]. In 2011, 120 000 people were estimated to have some degree of hearing loss in the Portuguese population [7]. The World Health Organization (WHO) defines as disabling hearing loss when it is higher than 40 dB in the better ear (Annex A) [6].

HL may be genetic, caused by single gene mutation or by a combinations of mutations in various genes, environmental, caused by trauma, medication, medical problems or environmental exposure, also it can be due to an association between genetic and environmental. HL can also be syndromic or non-syndromic. Non-syndromic hearing loss (NSHL) can be inherited by an autosomal dominant mode, usually related to a delayed onset; an autosomal recessive, usually congenital and also mitochondrial and X-linked [8]. Syndromic HL is accompanied by additional clinical features, such as anomalies of the eye, kidney, the musculoskeletal and the nervous systems and pigmentary disorders [9].

HL can be present due to various dysfunctions and impairments in the inner ear, such as: impairment of stereociliar function, related to Usher syndrome [10]; enlargement of the cochlear aqueduct, present, for example in Pendred syndrome [11]; thickening of the *stria vascularis*, related to Alport syndrome [12]; acoustic trauma, causing noise-induced HL. Also HL can be idiopathic, such as sudden deafness or caused by ototoxic medication, such as tuberculostatics, salicylic acid and aminoglycosides, that alters the function of inner ear structures and alter fine tuning of mechanoelectrical transduction [5].

Tinnitus is the perception of a ringing tone within the ear without an external sound trigger. Tinnitus may be caused by alteration in the inner ear or auditory pathway or by vascular and muscular disorders, osseous disease or neoplasia. When the efferent system is affected by inner ear stressors it may cause tinnitus generation and maintenance [5].

1.2.1. Age-related Hearing Loss

Age-related hearing loss (ARHL) or presbycusis is a bilateral, progressive, mainly sensorineural hearing loss that occurs due to degenerative changes related to ageing of the cochlea and central auditory pathways, leading to high-frequency hearing loss and loss of speech discrimination [13–15]. ARHL results from the cumulative effect of intrinsic and extrinsic factors, such as loud noise exposure, medical disorders, medications, anatomical degeneration and genetic susceptibility [13,15].

ARHL shows considerable variation in age of onset, progression and severity [14]. Currently, it affects over 70% of the world population over 65 years old [16]. In 2015, there where 2,122,966 people over 65 years old, in Portugal [17]. United Nations projections show that the world's elderly population (over 60 years old) will double by 2050, surpassing 2 billion people [18]. This comes to show that ARHL will continue to be a growing public health problem in the coming years.

Genetic pre-disposition to ARHL is supported by studies conducted in mice [19–21]. It was found that 19 out of 80 inbred mouse strains showed early-onset hearing loss, while 16 of them revealed hearing loss at an older age. Further studies revealed 18 ARHL loci and 6 of the affected genes in mice have

been identified [19–21]. The identification of mutations causing hearing loss in humans and their genetic screening is now practical and carried out in some clinical environments. Knowledge of these mutations will also allow susceptible individuals to have a protective behaviour towards their hearing [21].

ARHL results in difficulty interpreting speech sounds, leading to a reduced ability to communicate. This translates into a physical and social impairment such as cognitive deficits, social isolation, dependence, frustration and reduced quality of life [22,23].

Histopathologically, ARHL has been classified as sensory, neural, strial, metabolic, cochlear-conductive, mixed or undetermined, based on temporal bone degeneration, age-related changes of the organ of Corti, ganglion cell, *stria vascularis* and basilar membrane and its correlation to audiometric data [24].

Nowadays, ARHL rehabilitation relies mostly on the use of hearing aids, which focus on the peripheral auditory part. However, the success rate is limited due to the diversity of structural changes in the hearing pathway. People with peripheral deficits show the better response to hearing aids. Cochlear implant also shows satisfying results in cases of profound hearing loss [24] where the auditory pathways is still preserved. New targeted treatments may be developed through the understanding of the genetics of ARHL, as it may be indicative of the biological pathways of ARHL.

1.2.1.1. GRM7 gene

Glutamate metabotrophic receptor 7 (encoded by *GRM7* gene), belongs to the family of G protein-coupled receptors and is linked to the inhibition of the cyclic AMP cascade. L-glutamate is a fast excitatory neurotransmitter, that activates both ionotropic and metabotropic glutamate receptors. It also presents excitotoxic properties in the central nervous system, when it is released in excess or its recycling mechanism breaks down. This leads to a excitotoxic swelling of the postsynaptic target, due to a massive entry of cations and water, possibly resulting in postsynaptic neuron degeneration and loss of internal calcium homeostasis [25,26].

In the mammalian cochlea, the best candidate for a neurotransmitter function at the synapses between the inner hair cells and the primary auditory neuron (type I spiral ganglion neurons) is glutamate. AMPA/kainate receptors are activated first and could be accountable for the synaptic transmission at low and moderate intensities and NMDA receptors seem to be secondarily activated by high-intensity sounds [27].

The single-nucleotide variant or SNP rs11928865, located in the *GRM7* gene, has been associated with ARHI in genome-association studies European and Chinese descendant populations [28,29]. This gene is thought to be central to maintain glutamate synaptic transmission and homeostasis at the synapses between hair cells and the dendrites of afferent auditory nerve fibers. Excessive quantity of glutamate could be the mechanism mediating neurotoxicity in auditory neurons [30].

1.2.1.2. NAT2 gene

Oxidative stress results from the accumulation of damage, due to an imbalance between the production and the removal of reactive oxygen species (ROS) and free radicals. Oxidative stress has an essential role in the ageing process and the diseases associated with ageing, such as ARHL [13,31].

During the lifetime, the inner ear suffers a series of insults, for instance exposure to noise, drug influences, ear diseases and age related degeneration, which allied with a specific genetic background, such as antioxidant enzymes polymorphisms, might increase ROS levels, leading to hair cell damage [13,31].

In the adult cochlea there are numerous antioxidant enzymes, such as glutathione S-transferase, catalase and N-acetyltransferase. *NAT2* (N-acetyltransferase 2) gene, located at chromosome 8p22,

codifies a N-acetyltransferase enzyme that detoxifies exogenic substrates by N–acetylation or O-acetylation, contributing to the balance of oxidative status. *NAT2* is highly polymorphic, resulting in intermediate, slow or rapid acetylators, having the latter two links to disease. For example, the missense substitutions of the *NAT2* alleles, G191A (NAT2*14A), T341C (NAT2*5A), G590A (NAT2*6A) and/or G857A (NAT2*7A), are associated with slow acetylator phenotypes. These missense substitutions result in different functional characteristics, such as decreased protein expression (NAT2*5A and NAT2*6A) and reduced protein stability (NAT2*14A and NAT2*7A). Previous studies have linked the NAT2*6A polymorphism to ARHL in the Caucasian population [13,31].

1.2.1.3. Mitochondrial DNA

The mitochondria is vital in various cellular processes such as ATP production, β-oxidation of fatty acids, iron-sulphur cluster synthesis and apoptosis. The mammalian mitochondria harbour their own genome, that encodes 13 proteins , 2 rRNAs and 22 tRNAs [32].

Mitochondrial DNA (mtDNA) mutations can lead to cellular energy conversion impairment and tissue dysfunction, being also tied to the pathophysiology of age-associated diseases, such as ARHL, and the ageing process itself. Morphology, abundance and oxidative phosphorylation activity changes are seen in ageing mitochondria [32]. Recently, a study by Falah *et al* has described mtDNA copy number as a predictive biomarker for ARHL, as subjects with ARHL present lower mtDNA copy number than healthy individuals, which suggests that it occurs through alteration of mitochondrial function [33].

Age-related mtDNA mutations increases in multiple human tissues such as heart, colon, skeletal muscle and brain. The spectrum of mutations is similar to nuclear DNA, including point mutations, insertions and deletions. A common age-related deletion mtDNA4977 deletion is usually associated with ageing. The levels of this mutation in human cochlear tissue was found to correlate with ARHL severity [32,34].

mtDNA allows the division of the human population in ethnic groups, based on several discrete haplogroups. Haplogroups emerged due to the uniparental inheritance pattern of mtDNA and the negligible recombination at the population level, subdividing the human population through mutations acquired over time. The major haplogroups arose 40000-150000 years ago and have defined different human populations as they migrated out of Africa. The major European haplogroups (H, J, T and U) emerged 39000-510000 years ago. Currently, there are 20 haplogroups identified in the European community [35,36].

It has been suggested that the presence of these “neutral” polymorphisms in mtDNA, defining different haplogroups might cause mild deleterious bioenergenic abnormalities, which contribute to the ageing process. A study by Manwaring *et al* found that haplogroups U and K (typically European) have a higher predisposition to ARHL [37,38]. On the other hand, a study conducted by Bonneux *et al* found no significant association between any haplogroups and ARHL [38,39].

1.3.Vestibular disorders

Vestibular disorders (VD) are considered a group of diseases leading to transient or permanent loss of vestibular function [3].

VD have a high clinical heterogeneity and overlapping symptoms among the different VD, which limits clinical characterization and phenotyping. According to their clinical presentation, three major syndromes can be distinguished: a) an **episodic** vestibular syndrome, such as motion sickness and vestibular migraine (VM); b) a **progressive** vestibular syndrome leading to bilateral vestibular hypofunction, including some rare disorders such as the cerebellar ataxia, neuropathy, vestibular areflexia syndrome (CANVAS); c) and diseases which **include sensorineural hearing loss** (SNHL) with a variable vestibular dysfunction, such as Meniere’s disease (MD) [3].

1.3.1. Meniere's Disease

MD is a multifactorial disorder that has its age of onset probably determined by the combined effect of genetics and environmental factors. Clinically, it is characterized by attacks of spontaneous vertigo usually associated with unilateral fluctuating SNHL, tinnitus and aural fullness. Vertigo attacks are typically more common in the first years of the disease, however HL and vestibular hypofunction are highly variable among patients. These patients can also develop bilateral SNHL, migraine, and systemic autoimmune diseases. This variability in the course of the disease makes MD phenotyping difficult [40].

The prevalence of MD is highly different depending of the population in study, ranging from 34-190 per 100,000 [40]. MD has a higher predominance in women and its age of onset ranges from the third to the seventh decades of life [40–42].

Even though MD is considered multifactorial, there are three main hypothesis for its cause: genetic, autoimmunity and allergy.

The frequency of familial cases in European population is 5-15% and in Asian population (South Korean) 6.3%. Familial MD (FMD) studies suggest a genetic heterogeneity and an autosomal dominant pattern of inheritance with incomplete penetrance, however families with recessive inheritance have also been described. FMD is associated with an earlier onset and a tendency to more severe symptoms in successive generations [43,44].

Several studies support that autoimmune mechanisms are associated to the pathophysiology of MD, such as: the finding of elevated levels of autoantibodies or circulating immune complexes in the serum of some patients [45–47]; the association with alleles of *DRB1* gene of the MHC reported in different populations; the association with a functional variant of LYP, a lymphoid protein phosphatase, which inhibits T cell receptors response in patients with bilateral ear involvement [48,49]; and inner ear antigens with molecular weights of 28, 42, 58 and 68 kDa could be the main components inducing autoimmune MD in guinea pigs [50].

The prevalence of diagnosed allergy is three times higher in MD patients than in the general population, being both inhalant and food allergens associated to MD [51]. Furthermore, MD patients have elevated levels of IgE (41.3%) compared to healthy controls (19.5%) [52].

Derebery and Berliner described three theories relating allergy to MD that focus on inflammation within the endolymphatic sac (ES): 1) the ES fenestrated blood vessels could allow antigen entry, causing **mast cell degranulation and inflammation**; 2) circulating immune complexes may be deposited in the *stria vascularis*, resulting in an **increased vascular permeability and fluid imbalance**; 3) **viral antigen-allergic interaction**, as viruses can damage epithelial surfaces, allowing an increase in antigen entry and increasing responsiveness to histamine [53].

In support of the theory that the ES is a target for allergic activity, a study conducted in human tissue, has detected the presence of histamine receptor HRH1 in the epithelial lining of the ES and HRH3 expressed exclusively in the sub-epithelial capillary network [54].

There is not an effective treatment for MD, however patients may be helped by a combination of lifestyle and dietary changes, as well as medical therapy (drugs, surgery and physiotherapy) and psychological counselling. Some of the suggested lifestyle changes are allergy-avoidance, reduction of salt intake, avoidance of caffeine, tobacco and alcohol. Regarding medical therapy, several drugs may be administrated, such as: diuretics, betahistine, steroid therapy, that may be used to treat acute and chronic symptoms and gentamicin intratympanic injection, a destructive treatment that can be used in patients with intractable vertigo [55–57].

1.3.2. Vestibular migraine

VM is defined by the occurrence of episodic vestibular symptoms and a history of migraine, with a temporal association in at least 50% of the attacks (Annex B) [58]. However, it has only been considered a distinct disease since the new millennia, due to its high symptom overlapping with MD [3,59].

VM has a population prevalence of about 1%, however it is considered underdiagnosed. The mean age of first occurrence is 37.7 years for women and 42.4 years for men. It is also 1.5-5 times more frequent in women than men [3,60]. VM has been described in several families with an autosomal dominant inheritance [58].

VM patients have a lack of sleep quality and high levels of depression, which causes a general decrease of health-related quality of life [60].

VM patients have a favourable response to anti-migraine drugs, which supports an underlying migraine mechanism. However, this evidence is insufficient as it is based on uncontrolled clinical case series, thus the apparent efficacy may be due to confounding factors, such as placebo response, spontaneous improvement and multiple drug effect [58].

There are families presenting familial hemiplegic migraine (FHM) and common migraine, which suggests a shared pathophysiology, as does VM. FHM has been linked to mutations in three genes: *CACNA1A*, *ATP1A2* and *SCN1A*, which induce changes in the conductance of these voltage-gated ion channels. However, no evidence of an association with genes encoding ion channels linked to FHM has been found in VM. On the other hand, ATPase activity, leading to an increase in Na^+ concentration has been linked to migraine pathophysiology. Therefore, genetic variation of ion channels and ion transporters, which regulate fluid homeostasis may underlie susceptibility to VM [3,61].

Migraine is more common in patients with MD than in healthy controls. At times, VM and MD may be indistinguishable based only on medical history and symptoms, also it can present as a comorbidity. VM patients may present fluctuating hearing loss that does not progress to profound levels, tinnitus and aural pressure. Also, MD patients during attacks may experience migraine headaches, photophobia and migraine auras. Yet the pathophysiological relation between VM and MD is uncertain [58].

2. Objectives

The aim of this work is (1) to genetically characterize patients with ARHL, for *GRM7* and *NAT2* genes and mtDNA haplogroups and (2) to investigate mold-induced immune response in VM patients and (3) to compare it to the response from MD patients.

Determining ARHL genotypes could allow ARHL to be faced as an avoidable disease and not an obligatory age condition, also it could contribute to a molecular diagnostic and to estimate the risk of ARHL.

Defining the effect of allergenic extracts from *Penicillium* and *Aspergillus* in proinflammatory cytokines and gene expression profile in VM patients peripheral blood mononuclear cells (PBMC) may allow to distinguish VM patients from MD patients.

Both groups of results will lead to an increased knowledge on the genetic and environmental contribution to inner ear diseases, the main focus of this study.

3. Materials and Methods

3.1. Patient Cohorts

Two different patient cohorts were used: one for the study of ARHL and another for the study of VM and MD.

3.1.1. ARHL Cohort

A total of 483 Portuguese middle aged and elderly people were included in this study, with age comprised between 49 and 100 years old. The samples were obtained from institutions that have protocols with the Deafness Group – Translational and Biomedical Investigation Unit, BioISI, FCUL, such as senior universities and retirement homes. To avoid loss of anonymity, all individuals were coded with alphabet letters (PRE) and numbers, to distinguish them. Written informed consent was obtained from all participants.

All patients involved in this study underwent audiometric examination, to assess the degree of hearing loss and a clinical history was obtained. The clinical variables studied were: cardiovascular disease, hypertension, diabetes, high cholesterol, tinnitus, thyroid disease, ototoxic disease, smoking habits, oncological disease, noise exposure, difficulty hearing in noisy spaces and hearing loss. HL (dB) is determined considering the average HL for the tested frequencies (500 Hz, 1000 Hz, 2000 Hz, 4000 Hz) in the best ear.

3.1.2. VM & MD Cohort

A total of 73 samples from patients with VM and 115 samples from patients with MD were collected for this study. Recruitment was carried out in centres distributed over Spain. The experimental protocols of this study were approved by the Institutional Review Board in all participating hospitals and every patient signed a written informed consent.

All patients underwent a complete neuro-otological evaluation and clinical history was obtained. The clinical variables studied were: gender, time course of disease, age of onset, family history of MD, hearing stage at diagnosis, type of headache, history of autoimmune disease, familial MD, cardiovascular risk factors (high blood pressure, type 2 diabetes, dyslipidemia and smoking) and Tumarkin crisis. Hearing stage was defined as a as four-tone average of 500 Hz, 1000 Hz, 2000 Hz and 3000 Hz according to the American Academy of Otolaryngology Head and Neck Surgery (AAO-HNS) (stage 1, $\leq$ 25 dB; stage 2, 26-40 dB; stage 3, 41-70 dB and stage 4, >70 dB). The types of headache are migraine and tension-type headache. Migraine can be accompanied by or without aura.

3.2. gDNA Extraction

gDNA was extracted from blood samples collected in FTA® cards (Whatman, GE Healthcare), the blood sample was collected through index finger skin puncture. FTA® cards were stored at room temperature. gDNA extraction protocol was followed by the NZY Tissue gDNA Isolation Kit (NZYTech, Lisbon) suppliers recommendations.

3.3.*HSV1* and *NAT2* PCR

The HVS1 of the mitochondrial genome was amplified and sequenced in order to identify polymorphisms. This region was amplified from position 15975 to 16515. In table 3.1, the primers used for the PCR reaction are identified.

Table 3.1- mtDNA primers and respective size of the amplicon.

Primer	Sequence	Amplicon size (bp)
HVS1-F	5'- CTCCACCATTAGCACCCAAAGC-3'	540
HVS1-R	5'- TGAAGTAGGAACCAGATGTCGG-3'	

The PCR reaction mix had the primers at 10 µM, KAPA2G Robust HotStart ReadyMix (2X) (0.5 U/25 µl of reaction) (KapaBiosystems, GrISP, Porto), sterile ultrapure water and DNA. In every PCR a negative control was added, without DNA, to confirm de absence of contaminants. The PCR conditions are present in annex B.

NAT2 gene was amplified and sequenced in order to identify seven SNPs: rs1799930, rs1799931, rs1801280, rs1208, rs1041983, rs1799929 and rs1801279, that allow to define a haplotype and the corresponding acetylation phenotype. *NAT2* gene was amplified from position 13886 to 14724, through a PCR reaction using the primers shown in table 3.2.

Table 3.2 – Primers used to amplify *NAT2* gene and respective amplicon size

Primer	Sequence	Amplicon size (bp)
NAT2-F	5'- CATTGTGGGCAAGCCA -3'	839
NAT2-R	5'- AACGTGAGGGTAGAGAGG -3'	

The PCR reaction mix had the primers at 10 µM, 2x Kapa 2G Fast Hot Start Ready Mix (0.5 U/25 µl of reaction) (KapaBiosystems, GrISP, Porto), sterile ultrapure water and DNA. In every PCR a negative control was added, without DNA. The PCR conditions are present in annex B.

All PCR products were observed in electrophoresis, with 2% agarose gel ((SeaKem® LE Agarose, Lonza) and in TBE buffer 0.5X. Midori Green Advanced DNA Stain (GrISP, Porto) was used as DNA intercalating agent, which is observable under blue light. The gel was photographed with the photographic system FastGene® FAS Digi (GrISP, Porto). To compare DNA fragment size it was used GRS Ladder 100 bp (GrISP, Porto). The loading buffer consisted of a 0.25% Bromophenol Blue, 0.25% Xylene cyanol, 95% Formamide and 10mM Sodium hydroxide solution.

3.4. Sequencing and Sequence Analysis

The PCR products were sent to a company STAB Vida, which purifies and sequences the samples. mtDNA sequences were analysed with Chromas Lite 2.1.1 software and compared to the reference sequence J01415.2, with NCBI's BLAST program. The mtDNA haplogroups were estimated by the mtDNA Haplogroup Analysis software [62].

NAT2 sequences were analysed with the alignment tool Mega6 and compared to the reference sequence NG_012246.1. *NAT2* haplotypes and genotypes were determined considering the N-acetyltranferases database. *NAT2* phenotypes were determined with NAT2PRED software [63].

3.5. Genotyping with Real-time PCR

The allelic discrimination of *GRM7*'s SNP rs11928865 was conducted by real-time PCR, with ABI StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The 10 µL reaction volume included 5 µL of TaqMan Genotyping Master Mix (Applied Biosystems), 0.5 µL of TaqMan Probe Mix (Applied Biosystems), 0.5 µL of ultrapure sterile water and 4 µL of DNA. The PCR conditions are in annex C.

3.6. PBMC Isolation and Stimulation

Peripheral blood was diluted 1:1 with 1× PBS and disposed carefully onto the corresponding 25:15 volume of Lymphosep, Lymphocyte Separation Media (Biowest, Nuaillé, France). Samples were centrifuged for 20 minutes at 2000 rpm to separate blood content. PBMC were collected and washed with 1× PBS and cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (Biowest, Nuaillé, France) and plated at $1.25x10^6$ cells/mL in 6-well plates. In some experiments, fungus extract was added (2 Allergenic Extract-Mix fungus containing, in one case, 4 equal parts of Mix *Aspergillus* (*oryzae*, *repens*, *niger* and *terreus*) and in the other case, 4 equal parts of Mix *Penicillium* (*brevicompactum*, *expansum*, *notatum* and *roqueforti*)). The combinations were dialyzed to remove phenol using Tube-o dialyzer (VWR International, Radnor, PA, USA) against deionized distilled water. Each subject had a well per condition and 1.25 x 10^6 PBMC/mL was stimulated with 5 µg/mL dialyzed mixed fungus. PBMC were incubated during 16 h at 37°C in 7% CO2 and compared with cultured, unstimulated PBMC. The optimal concentrations of these mixtures were identified for previous studies conducted by the group. At the end of all incubations, PBMC were centrifuged, RNA and proteins harvested and supernatants were collected and stored at -80 °C.

3.7. RNA Extraction

RNA was isolated using the RNeasy® Mini Kit (Qiagen) following the manufacturer's protocol. RNA concentration was measured on a Nanodrop (NanoDrop Technologies Inc.,Wilmington, DE, USA). RNA quality was checked using Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

3.8. Quantitative PCR

One-hundred ng of RNA was converted into cDNA using Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Fisher Scientific) and cDNA samples were diluted to 2 ng/µL for the qPCR assays.

The quantification of IL-1β, IL-1RN, IL-6, TNFα and Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH) gene expression was conducted by quantitative PCR (qPCR) from each cDNA sample. Reactions were made with a final primer (table 3.3) concentration of 1 µM in a final volume of 20 µL and using Brilliant III Ultra-Fast SYBR® GreenQPCR Master Mix (Agilent Technologies) as detection system under the PCR conditions present in table 8.5 (Annex C), using an ABI 7900 HT Fast Real-Time PCR Systems (Life Technologies). No template controls were included in all qPCR plates. To check for reproducibility, four technical replicates were carried out for each sample. GAPDH was used as an endogenous expression control.

Table 3.3 – Primers used to amplify cDNA of each cytokine and respective amplicon size.

Primer	Sequence	Amplicon size (bp)
IL1B_fw	5'- AATCTCCGACCACCACTACAG - 3'	217
IL1B_rv	5' – GTTCAGTGATCGTACAGGTGC – 3'	
IL6_fw	5' – CCACTCACCTCTTCAGAACGA – 3'	211
IL6_rv	5' – TGATTTTCACCAGGCAAGTCTC – 3'	
TNFa_fw	5' - TCTTCTCCTTCCTGATCGTGG – 3'	186
TNFa_rv	5' – GAGGGTTTGCTACAACATGGG – 3'	
IL1RN_fw	5' – GGATTCACAAGACGATCTGCC – 3'	220
IL1RN_rv	5' – ATCACCAGACTTGACACAGGA - 3'	
GAPDH_fw	5' – ATCACCATCTTCCAGGAGCGAGA – 3'	193
GAPDH_rv	5' – CATGGTTCACACCCATGACGAACA - 3'	

3.9. Extracellular Cytokine Level Determination

Conditioned supernatants were collected and stored at –80 ºC until a sufficient number of samples were acquired. Frozen samples were thawed immediately prior to analysis and none of the samples underwent repetitive freeze-thawing cycles prior to analysis. IL-1β, IL-6, IL-1RA and TNFα levels in conditioned supernatant were quantified simultaneously with a commercially available Multiplex Bead-Based Kit (EMD Millipore, Billerica, MA, USA), in accordance with the kit-specific protocols provided by Millipore, using a Luminex 200 (Luminex Corp., Austin, TX, USA) and read with Luminex x PONENT 3.1 software (Luminex Corp.). The minimum detection limit for the assay was 0.14pg/mL and the maximum 10000 pg/mL. Samples with readings below or above these levels were assigned values of 0pg/mL for the minimum value or 10000pg/mL for the maximum value. The reliability of the measurements were previously determined for previous studies conducted by the group, by calculation of the Intraclass Correlation Coefficient (ICC) and sandwich-ELISA (>80%).

3.10. Expression Array

Expression levels were measured using the HumanHT-12 v4 Expression BeadChip (Illumina Inc.) with 500 ng of total RNA and processed with the high resolution scanner iScan (Illumina Inc.). Limma R package from Bioconductor was used for expression data analysis, normalization and differential expression analysis.

Signalling pathway analysis was performed using Ingenuity Pathways Analysis (IPA®, Qiagen, Venlo, Netherlands, http://www.ingenuity.com/products/ipa) software. Core analysis tool was executed using the DEG with an adjusted p-value cut-off of 0.001.

3.11. Statistical Analysis

A descriptive analysis was conducted using SPSS software (SPSS Inc.) for both cohorts.

The audiograms were analyzed considering the best ear (estimated based on the lowest average of frequencies of 500 Hz, 1000 Hz, 2000 Hz and 4000 Hz). For the results from ARHL cohort, Chi-square Test was used for general association between two variables were used. Kruskal-Wallis (for more than 2 groups) tests were employed to compare hearing thresholds. All the results were analysed through logistic regression model, where age was considered as control for all the others variables.

For the results from VM and MD cohort, quantitative variables were compared using Student's unpaired T-test. Qualitative variables were compared using cross tables and Fisher's exact test.

The level of significance considered was p-value<0.05 for both cohorts.

4. Results

4.1. Demographic Results

A descriptive analysis was conducted for both cohorts. Demographic results from ARHL cohort were analysed considering the influence of the variables on hearing loss. VM and MD cohorts were compared to determine differences between the demographics of the diseases.

4.1.1. ARHL Cohort

Due to the nature of the questionnaire, it was not possible to obtain full information from the 483 individuals, therefore on table 4.1 the percentage of each variable is relative to the number of answers obtained. This cohort is composed mostly by women and the mean age is 76.4±9.8 years. A great majority of the individuals also show difficulty of listening in noisy environments (69.1%) and have tinnitus (68.5%).

Table 4.1 - ARHL cohort descriptive analysis of demographics and clinical history.

Variables	ARHL (N (%)
Age, mean (SD)	76.4 (9.8)
Sex (female %)	332 (70.2)
Noise exposure	135 (33.3)
Difficulty hearing in noisy spaces	268 (69.1)
Cardiovascular disease	115 (29.0)
Hypertension	250 (63.0)
Diabetes	106 (27.6)
High cholesterol	221 (57.1)
Tinnitus	258 (68.5)
Thyroid disease	61 (15.9)
Ototoxic disease	63 (21.6)
Smoker	95 (23.3)
Oncological disease	69 (16.6)

The effect of demographics and comorbidities was tested for each of the hearing frequency considered. It was evident that there was a significant difference for sex, age, hypertension and cardiovascular disease, as seen on table 4.2. The effect of sex it is evident on lower frequencies (250 Hz and 500 Hz) where men have better hearing and at 4000 Hz there is an inversion and women turn into the better listeners (figure 4.1).

They were then categorized by mean HL in the best ear according to the WHO classification, which revealed that 33% of the sample has normal hearing and 67% of the sample have hearing loss (34.5% have mild HL, 27.5% have moderate HL and 5% have severe to profound HL). The same characteristics where analysed to see if they have an effect on the prevalence of HL (Annex D), which showed that age, cholesterol, hypertension and cardiovascular disease have an effect on HL. Moreover, a multivariate logistic regression controlled by age was conducted, which showed the effect of cholesterol and diabetes on HL (table 4.3). This is, for the same age, people with cholesterol show a 40% inferior probability of HL compared to people without cholesterol. People with diabetes show a 77% superior probability of HL than people without diabetes

Table 4.2 - Effect of demographic and clinical features on HL per frequency (Hz)

Characteristic	p-value for each frequency					
	250 Hz	**500 Hz**	**1000 Hz**	**2000 Hz**	**4000 Hz**	**8000 Hz**
Sex	**0,032***	**0,028***	0,166	0,493	**0,001***	0,148
Age	**<0.001***	**<0.001***	**<0.001***	**<0.001***	**<0.001***	**<0.001***
Cholesterol	0,100	0,388	0,247	0,078	**0,017***	**0,013***
Hypertension	**0,005***	**0,001***	**0,005***	**0,018***	**0,020***	**0,020***
Tinnitus	0,204	0,163	0,532	0,757	0,803	0,683
Diabetes	0,246	0,838	0,321	0,201	0,203	0,801
Smoking	**0,010***	**0,045***	0,057	**0,041***	0,395	0,170
Cardiovascular Disease	**0,004***	**0,002***	**0,018***	0,057	0,491	0,331
Noise Exposure	0,392	0,592	0,618	0,682	0,164	0,438
Ototoxic Medication	0,284	0,196	0,428	0,210	0,392	0,126

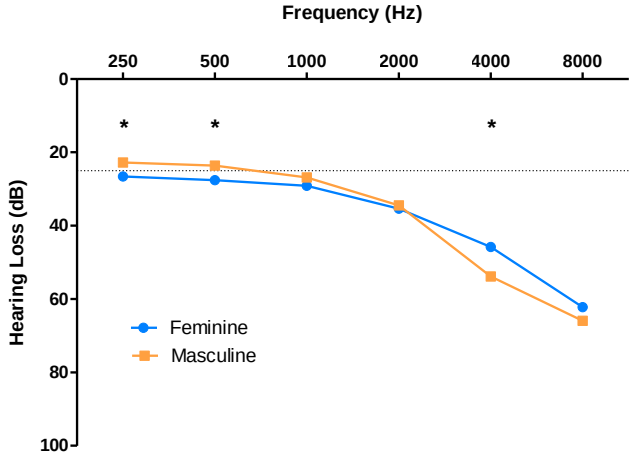

Figure 4.1 - Effect of sex on HL (dB) for each tested frequency (Hz)

Table 4.3 - Multivariate logistic regression controlled by age.

Characteristic	OR	p-value
Sex (Masculine)	1.34	0.294
Cholesterol (Yes)	**0.60**	**0.077***
Hypertension (Yes)	1.23	0.487
Tinnitus (Yes)	1.14	0.664
Diabetes (Yes)	**1.77**	**0.089***
Smoking (Yes)	1.29	0.438
Cardiovascular Disease (Yes)	1.51	0.225
Noise Exposure (Yes)	1.46	0.203
Ototoxic Medication (Yes)	0.92	0.831

4.1.2. VM & MD cohort

It was not possible to obtain full information from all the patients, therefore on table 4.4 and table 4.5 the percentage of each variable is relative to the number of answers obtained.

VM patients have a mean age of onset of 37.1±15.0 and have slightly more frequent migraine with aura. These patients have no history of Tumarkin crisis (table 4.4). On the other hand, MD patients have a higher age of onset (48.3±14.4 years) and have least frequently headaches, also they have a slight predominance of female affected patients (table 4.5).

Table 4.4 - VM patients' descriptive analysis for demographics and clinical history.

Variables	**VM**
Age, mean±SD	48.2±15.0
Sex (female) n (%)	51 (75)
Age of onset (SD)	37.1 (17.1)
Age of onset <40, n(%)	41 (60.3)
Time course (years), mean±SD	10.4±9.8
Headache (%)	67 (98.5)
Type of headache (migraine), n (%)	64 (94.1)
Type of migraine (with aura), n (%)	28 (57.1)
Smoking, n (%)	13 (19.1)
High blood pressure, n (%)	18 (32.7)
Tumarkin crisis, n (%)	0 (0)

Table 4.5 - MD patients' descriptive analysis for demographics and clinical history.

Variables	**MD**
Age, mean±SD	60.7±12.6
Sex (female %)	67 (58.8)
Age of onset±SD	48.3±14.4
Age of onset <40, n(%)	32 (28.6)
Time course (years), mean±SD	10.3±9.0
Affect ear (unilateral) n (%)	73 (64.0)
Headache (%)	42 (39.6)
Type of headache (migraine), n (%)	22 (48.9)
Familial md, n (%)	10 (9.7)
Hearing stage	
1	9 (8.2)
2	31 (28.2)
3	51 (46.4)
4	19 (17.3)
Autoimmune disease (%)	16 (17.6)
Smoking, n (%)	17 (16.7)
High blood pressure, n (%)	26 (26.5)
Dyslipemia, n(%)	21 (27.6)
Type 2 diabetes	9 (9.8)
Tumarkin crisis, n (%)	18 (17.6)

A comparison between VM and MD patients revealed that there are differences between the age of the patients, age of onset of the disease, prevalence of females affected, frequency of headaches and migraine (table 4.6). However, when comparing VM to the specific MD type 4 [64], characterized by the presence of migraine in all cases, it is observed that there are only significant differences in age of the patients and the age of onset of the diseases (table 4.6).

Table 4.6 - Comparison of VM patients' demographics and clinical history to MD patients and MD type 4 patients.

Variables	VM	MD	p-value	MD type 4	p-value
Age, mean±SD	48.2±15.0	60.7±12.6	**1.18×10^{-8}**	60.7±12.2	**0.0104**
Sex (female %)	51 (75)	67 (58.8)	**0.023**	9 (81.8)	0.476
Age of onset±SD	37.1±17.1	48.3±14.4	**5.0×10^{-6}**	51.9±13.1	**0.00760**
Time course (years), mean±SD	10.4±9.8	10.3±9.0	0.960	5.6±5.1	0.121
Headache (%)	67 (98.5)	42 (39.6)	**8.18×10^{-18}**	11 (100)	0.861
Type of headache (migraine), n (%)	64 (94.1)	22 (48.9)	**4.2×10^{-8}**	11 (100)	0.542

4.2. *GRM7* gene

From the total of 467 people genotyped by real-time PCR for the *GRM7* SNP rs11928865, only 337 individuals had full audiogram information, therefore only these were considered for the analysis. In table 4.7, it is possible to observe that the most common genotype is T/T with a corresponding mean HL of 35.01±15.61 dB and the least frequent genotype is A/A with a mean HL of 29.70±16.03 dB.

The genotype effect was tested for each tested hearing frequency and as observed in figure 4.2, there is a significant difference at 8000 Hz (p-value=0.048). However, when categorized by mean HL in the best ear according to the WHO classification and tested to evaluate the effect of *GRM7* SNP rs11928865 genotype on HL there were no significant differences (p-value=0.133). Also, a multivariate logistic regression controlled by age showed that comparing to A/A genotype, A/T genotype had a 1.93 times higher hearing loss risk and T/T genotype a 2.08 times higher hearing loss risk, but this was not statistically different (table 4.8).

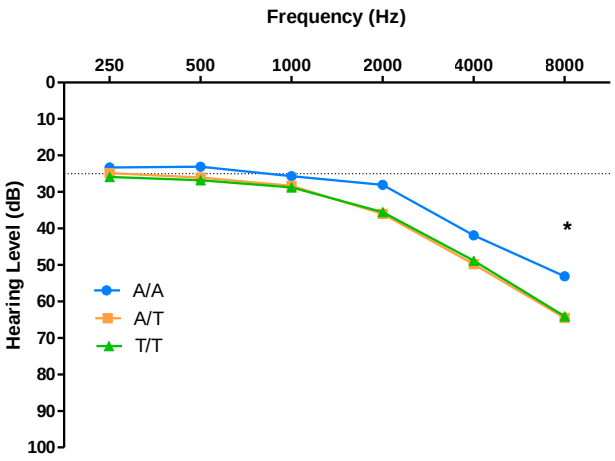

Figure 4.2 – Effect of *GRM7* SNP rs11928865 genotype on HL (dB) for each tested frequency (Hz).

Table 4.7- Distribution of individuals according to GRM7 rs11928865 and its associated mean HL ± Standard deviation (SD).

Genotype	N (frequency)	Mean HL± SD (dB)
A/A	21 (6.2%)	29.70±16.03
A/T	115 (34.1%)	33.95±16.01
T/T	201 (59.6%)	35.01±15.61

Table 4.8 - Logistic model applied to HL controlled by age, considering GRM7 SNP rs11928865 A/A genotype as reference

GRM7 genotype	OR	(95% CI)	p- value (Wald)
A/T	1.93	(0.69,5.52)	0.210
T/T	2.08	(0.77,5.68)	0.147

4.3. NAT2 gene

From the total of 442 people genotyped by real-time PCR for 7 SNPs of *NAT2:* rs1799930, rs1799931, rs1801280, rs1208, rs1041983, rs1799929 and rs1801279, only 334 individuals had full audiogram information, therefore only these were considered for the analysis.

The most common *NAT2* haplotypes are NAT2*4/NAT2*5U (21%) and NAT2*4/NAT2*5B (18%) (annex E). As some of the haplotypes are not sufficiently represented to conduct a statistical analysis, we only considered the ones that were present in at least 10 individuals. It was observed a statistical difference (p-value=0.037) between *NAT2* haplotypes at a 250 Hz frequency. When analysed more deeply it was determined that this difference is found between haplotypes NAT2*4/NAT2*6A and NAT2*5B/NAT2*5B (adjusted p-value=0.012). Likewise, a multivariate logistic regression controlled by age showed that there was a statistically significant difference for NAT2*4/NAT2*5A, when using NAT2*6A/NAT2*6A as reference, as seen on table 4.9. However, when tested using the WHO classification, no statistical differences were found (p-value= 0.963).

In table 4.10, it is possible to observe that an higher HL belongs to a slow acetylation phenotype and a lower HL belongs to a rapid acetylation phenotype, however no statistically significant differences were found when testing for each frequency independently (p-value=[0.309;0.635]) or for HL by the WHO classification (p-value=0.639). Likewise, a multivariate logistic regression controlled by age showed no differences (for R p-value=0.540 and for S p-value=0.652).

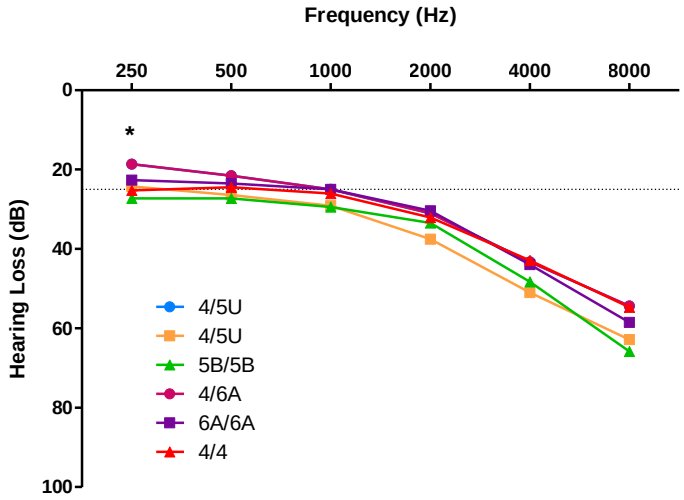

Figure 4.3 - Effect of NAT2 haplotypes on HL (dB) for each tested frequency (Hz).

Table 4.9 - Logistic model applied to HL controlled by age, considering the haplotype NAT2*6A/NAT2*6A as reference

NAT2 haplotype	OR	(95% CI)	p- value (Wald)
4/4	0.180	(0.34, 4.21)	0.079
4/5A	-0.073	(0.17, 5.19)	**0.007**
4/5B	0.390	(0.55, 3.99)	0.589
4/5U	0.404	(0.53, 4.21)	0.588
4/6A	0.185	(0.43, 3.34)	0.126
5B/5B	0.404	(0.55, 4.07)	0.628

Table 4.10 - Distribution of individuals according to *NAT2* phenotype and associated mean HL±SD.

Phenotype	N (frequency)	Mean HL±SD (dB)
Slow (S)	171 (51.19%)	34.79±15.88
Intermediate (I)	140 (41.92%)	33.54±15.59
Rapid (R)	23 (6.89%)	30.71±15.17

4.4.mtDNA

From the total of 444 people's DNA sequenced for HVS1 of mtDNA, only 336 individuals had full audiogram information and were considered for the analysis. The most common haplogroup detected in our sample was haplogroup H and the least common haplogroup M and haplogroup Y (annex F). As some of the haplogroups are not sufficiently represented to conduct a statistical analysis, we only considered the ones that were present in at least 12 individuals. On table 8.8 (Annex F), it is possible to observed that of the tested haplogroups, the one with the highest hearing loss is haplogroup T and the one with the lowest hearing loss is haplogroup L, yet no statistically significant differences were found when testing for each frequency independently (p-value=[0.136;0.747]) or for HL by the WHO classification (p-value=0.979).

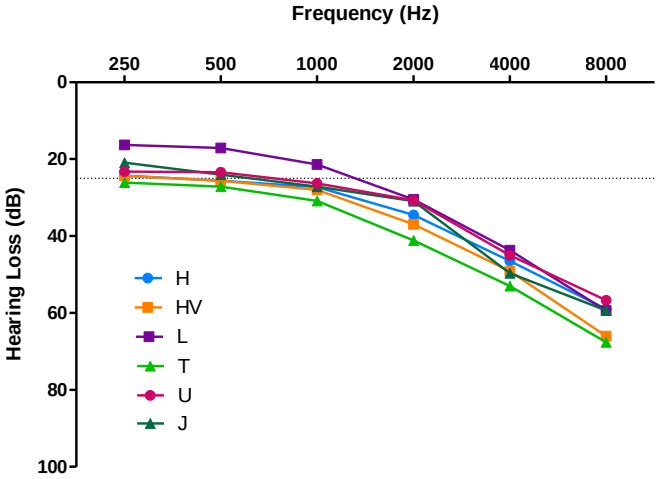

Figure 4.4- Hearing loss of the most common haplogroups by tested frequencies.

4.5. qPCR

qPCR was conducted to determine the expression levels of IL-1β, IL-1RN, IL-6 and TNFα genes in VM patients and healthy controls.

Fold-change values of cytokine expression levels were determined between stimulated and non-stimulated PBMC of controls and VM patients. As seen in table 4.11, VM patients present higher fold-change values for IL-1, IL-1RN and IL-6, but not for TNFα, although none of them are significantly different.

Fold-change values of cytokine expression levels between VM patients and controls were calculated, and as observed in figure 4.5 when stimulating PBMC with *Aspergillus* there is a tendency for an higher expression of IL-1, TNFα and IL-6, being the last significantly different. Furthermore, when stimulating PBMC with *Penicillium* there is not a change in the expression levels of either cytokines.

Table 4.11 –Fold-change values for cytokine expression levels between stimulated and non-stimulated PBMC of controls and VM patients.

Cytokine	Stimulation	Case	mean $2^{-\Delta\Delta Ct}$	p-value
IL-1	*Aspergillus*	Control	2,55E+02	0.631
		VM	9,40E+02	
	Penicillium	Control	2,06E+02	0.536
		VM	6,00E+02	
IL-1RN	*Aspergillus*	Control	4,52E+02	0.604
		VM	7,00E+08	
	Penicillium	Control	1,89E+02	0.940
		VM	2,03E+02	
IL-6	*Aspergillus*	Control	5,08E+01	0.053
		VM	9,51E+02	
	Penicillium	Control	8,43E+02	0.817
		VM	1,04E+03	
TNFα	*Aspergillus*	Control	2,20E-01	0.061
		VM	1,97E+00	
	Penicillium	Control	3,41E-01	0.266
		VM	1,33E+00	

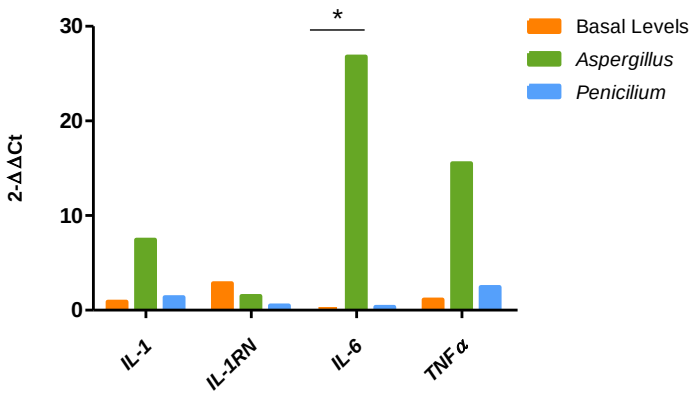

Figure 4.5 - Fold-change values for cytokine expression levels between patients with VM and controls. * - p-value=0.048.

4.6. Extracellular Cytokine Levels

The level of IL-1β, IL-1RA, IL-6 and TNFα present in the cells supernatant was determined using a commercially available Multiplex Bead-Based Kit.

IL-1RA levels were significantly higher in VM patients than in controls (figure 4.6A). IL-6 and TNFα levels were significantly higher in VM patients when compared to controls, with both stimulations (*Aspergillus* and *Penicillium*), however the difference is significantly higher when stimulated with *Penicillium* (figure 4.6B and 4.6C).

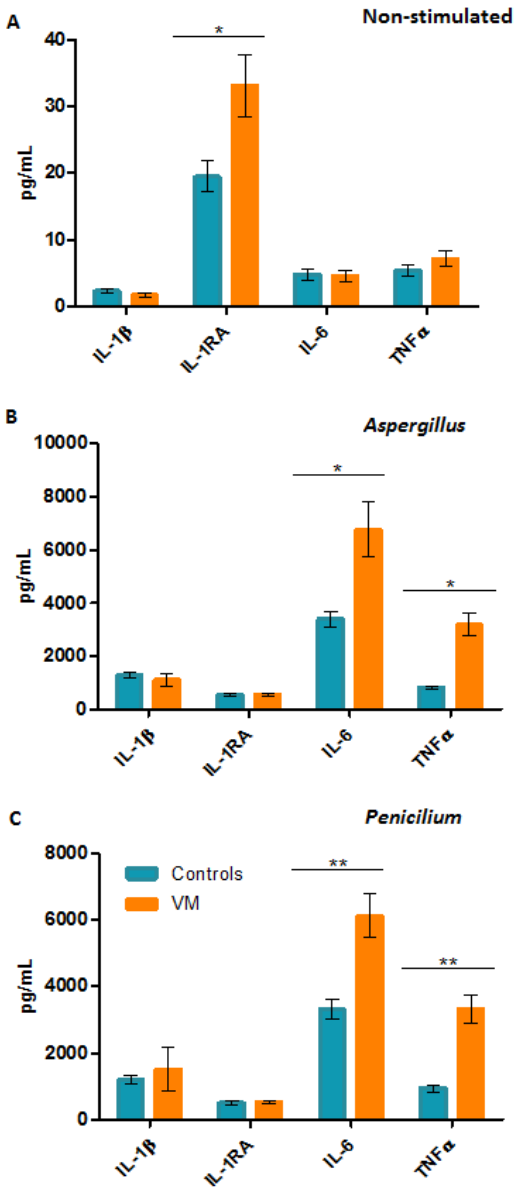

Figure 4.6- Cytokine levels in VM patients and controls. A- basal levels; B- stimulated with *Aspergillus*; C- stimulated with *Penicillium*
* - p-value < 1×10^{-3}; ** - p-value [1×10^{-3} - 1×10^{-5}];
*** - p-value > 1×10^{-5}

Previous studies in our group have determined that MD patients can be grouped according to their IL-1β and TNFα levels. With this knowledge, we decided to compare VM patients with the different categories of MD patients (unpublished findings).

When comparing the basal levels of VM to MD with low IL-1β and TNFα levels, we can observe that there is only a significant difference between VM and MD with low levels of TNFα in IL-1RA (figure 4.7A).

When comparing the stimulated levels (with *Aspergillus* and *Penicillium*) of VM to MD with low IL-1β and TNFα levels, we can observe that there is a significant difference in the levels of IL-1RA and IL-6 (figure 4.7B and 4.7C).

From the comparison of the basal levels of VM to MD with high IL-1β and TNFα levels, we can observe that there is a significant difference between the levels of all evaluated cytokines (Figure 4.7D). However, the stimulated levels with *Aspergillus* are only significantly different when comparing the levels of IL-1RA of VM and MD with high TNFα levels (figure 4.7E). Also, the stimulated levels with *Penicillium* are significantly different when comparing the levels of IL-1RA and IL-6 of VM and MD with high TNFα levels (figure 4.7F).

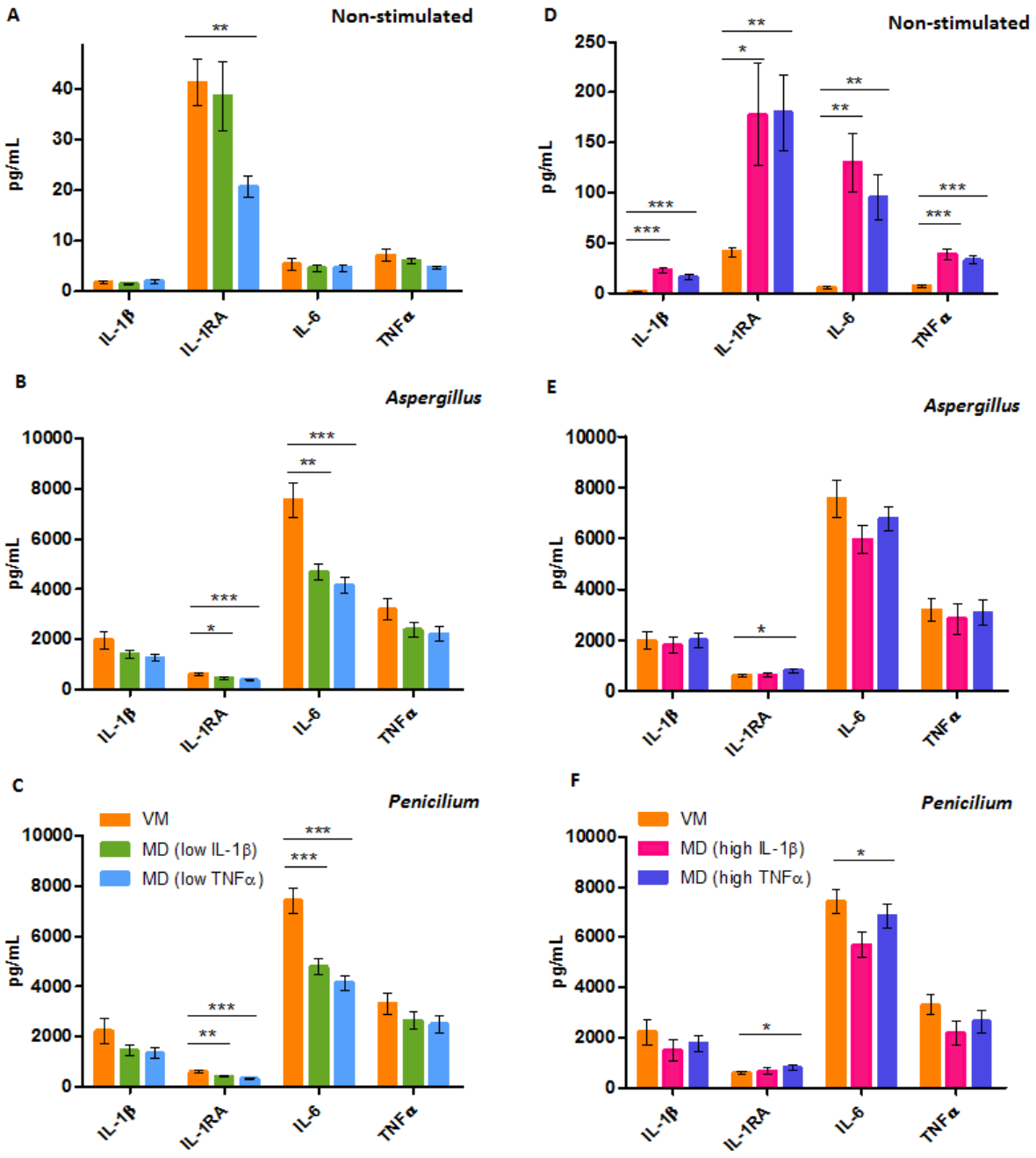

Figure 4.7 - Cytokine levels in VM patients and MD patients. A - basal levels (MD with low cytokine levels); B - stimulated with *Aspergillus* (MD with low cytokine levels); C - stimulated with *Penicillium* (MD with low cytokine levels); D - basal levels (MD with high cytokine levels); E - stimulated with *Aspergillus* (MD with high cytokine levels); F - stimulated with *Penicillium* (MD with high cytokine levels). * - p-value < 1×10^{-3}; ** - p-value [1×10^{-3} - 1×10^{-5}]; *** - p-value > 1×10^{-5}

4.7.Gene Expression

We compared gene expression profiles in non-treated PBMCs from VM patients with MD patients with high (↑) and low (↓) levels of cytokines and also with healthy controls. We performed a Core analysis from IPA® software using all genes from the expression profiles. The Upstream analysis within the Core analysis identifies upstream regulators that are predicted to be activated or inhibited based on an activation z-score. The comparisons between gene expression profiles in non-treated PBMCs of VM patients and MD patients with ↓-level of cytokines and also between VM patients and healthy controls revealed no statistically significant differences. However, there are statistically significant differences between VM patients and MD patients with ↑-level of cytokines. Based on the pathways retrieved, we could predict the involvement of B-cell maturation, cytokine production and dendritic cell maturation (table 4.12).

To further characterize the role of mold in inflammatory response, VM patients PBMCs were cultured and their responses to mold were directly compared with stimulated PBMC from healthy volunteers. Based on the pathways retrieved (table 4.13), we could predict that the stimulation by *Aspergillus* and *Penicillium* involves inflammation and cytokine production as well as apoptosis signalling.

Additionally, we compared the response from VM patients stimulated PBMC and MD patients stimulated PBMC (table 4.14 and table 4.15), which also revealed the involvement of upstream regulators that take part in differentiation, activation and regulation of T-cells, B-cell development, dendritic cell maturation and allergy.

Table 4.12 - Canonical pathways state in non-stimulated MD patients with ↑-level of cytokines compared to VM patients.

Canonical Pathway	**p-value**	**State**
CD28 Signaling in T Helper Cells	1.77×10^{-12}	Inhibited
iCOS-iCOSL Signaling in T Helper Cells	3.49×10^{-9}	Activated
Dendritic Cell Maturation	3.79×10^{-9}	Activated
PI3K Signaling in B Lymphocytes	3.87×10^{-9}	Inhibited

Table 4.13 - Canonical pathways involved in VM exclusive to stimulation with *Aspergillus* or *Penicillium.*

Pathway	***Aspergillus***		***Penicillium***	
	State	**p-value**	**State**	**p-value**
IL-8 Signaling	Inhibited	$1{,}59\times10^{-7}$	Activated	6.59×10^{-9}
Death Receptor Signaling	Inhibited	$6{,}70\times10^{-7}$	Inhibited	4.05×10^{-7}
Toll-like Receptor Signaling	Inhibited	$7{,}76\times10^{-7}$	Inhibited	1.13×10^{-5}
NFkB Signaling	Inhibited	$8{,}46\times10^{-7}$	Inhibited	3.73×10^{-6}

Table 4.14 - State of upstream regulators in MD comparing to VM, exclusive to each stimulation (p-value< 10^{-5}).

Stimulation	Upstream Regulator	p-value	State
Aspergillus	RICTOR	3.82×10^{-7}	Inhibited
	TCR	1.32×10^{-11}	Activated
	XBP1	6.89×10^{-8}	
	E2F1	9.50×10^{-7}	
Penicillium	IL1RN	6.44×10^{-10}	Inhibited
	PTGER4	2.17×10^{-9}	
	TRIM24	2.10×10^{-8}	
	PRL	9.15×10^{-13}	Activated
	HGF	3.34×10^{-12}	
	IFNA2	1.00×10^{-11}	
	STAT1	4.97×10^{-11}	
	CD40LG	7.90×10^{-11}	
	TLR3	3.05×10^{-10}	
	IRF3	5.63×10^{-10}	

Table 4.15 - State of upstream regulators in MD comparing to VM, common to both stimulations (p-value<10^{-5}).

Upstream Regulator	p-value (*Aspergillus*)	p-value (*Penicillium*)	State
IFNG	2.07×10^{-14}	3.03×10^{-27}	Activated
TNF	1.40×10^{-15}	9.18×10^{-28}	
MYC	4.94×10^{-10}	2.9×10^{-11}	
IL2	5.19×10^{-11}	5.54×10^{-23}	
NFkB (complex)	1.62×10^{-9}	7.69×10^{-7}	
TLR4	2.47×10^{-8}	1.63×10^{-14}	
IL1A	1.04×10^{-7}	1.28×10^{-7}	
HIF1A	1.96×10^{-7}	1.27×10^{-10}	
Interferon alpha	1.06×10^{-6}	1.90×10^{-15}	
GFI1	3.02×10^{-7}	3.33×10^{-10}	Inhibited
CD3	2.00×10^{-6}	4.45×10^{-11}	
CD28	2.76×10^{-6}	1.34×10^{-9}	

5. Discussion

The present work is focused in different aspects related to inner ear diseases in order to achieve a better knowledge on it. The first part of this work, considers a sample of individuals with ARHI in the Portuguese population and the second part considers a sample individuals with vestibular episodic syndrome (VM and MD) from the Spanish population.

The individuals of the Portuguese sample underwent a clinical history questionnaire, hearing evaluation and genetic screening analysis. These individuals were middle aged and elderly people aged between 49 and 100 years, screened in an attempt to find factors that might contribute to the identification of risk factors and consequently to the diagnosis of ARHL and inform future therapeutic interventions.

Our results show that there are significant differences in HL for age, for each frequency and overall HL (table 4.2 and table 4.3), as expected. A study by Lin *et al* (2011) in the United States of America population, reported a HL prevalence of 63.1% in participants over 70 years [65]. Considering the increase in life expectancy, these results present obvious consequences of ageing.

We found that men have significantly better hearing for lower frequencies (250 Hz and 500 Hz) and women at high frequencies (4000 Hz), in concordance with previous results. Lin *et al* (2011) studied the association between sex and HL applying different frequency ranges: standard [500–2000 Hz] speech [500–4000 Hz] and high frequency [3000–8000 Hz] and found that male sex was associated to greater hearing loss at speech frequency and high frequencies [65]. On the other hand, a study by Homans *et al* (2016) found that men had overall significantly better thresholds at the lower frequencies, whereas women had overall significantly better thresholds at the higher frequencies [66], which is in agreement with our findings since we observe an gender reversal phenomenon of ARHL. This may be explained by the fact that women have a lifestyle that is more similar to men in comparison with 20 to 30 years ago, suggesting that the environment and lifestyle have an essential effect on the development of ARHL [66].

Distinct types of comorbidities have been related to presbycusis. When these were studied for the influence in each hearing frequency, we found that cholesterol (4000 Hz and 8000 Hz), hypertension (250 Hz-8000 Hz), smoking (250 Hz, 500 Hz and 2000 Hz) and cardiovascular disease (250 Hz-1000 Hz) had effect on HL. However, when adjusting the data to a multivariant model controlled by age, it is only observed a statistically significant difference for cholesterol and diabetes: patients with high cholesterol levels have a 40% lower probability of HL and patients with diabetes have a 77% higher probability of HL, for patients with the same age.

Lee *et al* (2013) and Sogebi *et al* (2017) reported that smoking had a positive correlation with HL, which is in agreement with our findings [67,68]. However, reports that smoking tobacco have no effects on HL have also been made by Bener *et al* (2016) [69], but on the other hand they found a relation between high levels of cholesterol and HL[69].

Our results on the effect of cholesterol and HL could also be related with specific medication for the pathology and not directly with lipid concentration. Gopinath *et al* (2011) found that serum lipids were not associated to prevalence of HL, however people using statin (cholesterol lowering medication) were 48% less likely to have HL compare to those not using the treatment [70]. On the other hand, Lee *et al* (2015) concluded that their study partially supported the absence of a relevant relationship between high frequency HL and low high density lipoprotein- cholesterol, as they only found a small association [71]. Anatomically, high cholesterol affects the inner ear blood supply and the lateral wall stiffness of the cochlear outer hair cells, therefore further studies should be conducted to assess the effect of cholesterol on ARHL, including very high frequency HL (8-16 kHz) [71,72].

Various studies, conducted in very different settings, such as a Nigerian population, an highly endogamous population and an American population have described being hypertensive and diabetic as risk factors for HL, which support the finding of this study [65,67,69,73].

Hutchinson *et al* (2010) found that old adults with high cardiovascular health had the best hearing ability, suggesting the protective effect of cardiovascular fitness on hearing [74]. Additionally, McKee *et al* (2017) as described an association between cardiovascular disease and ARHL [75], supporting our results.

As ARHL and hypertension are common and widespread disorders, especially in elderly subjects is not surprising that hypertension, as well as cardiovascular disease may influence ARHL, thus cardiologists should be add to the multidisciplinary team of professionals working to improve quality of life for these patients through a dynamic therapy process.

The study of the association of demographic variables and clinical history of our subjects to ARHL has had various limitations, being the most impacting the incomplete questionnaires and the inability to obtain the audiometric data, usually due to cerumen occlusion. Also, smoking habits information is obtained as "current smoker"/"non smoker", which makes us loose information about people who could have smoked but no longer do and this might influence the results.

ARHL is thought to have an environmental and genetic component [13,15]. It is estimated that 35-55% of auditory ageing has a genetic element [76].

As previously stated, *GRM7* SNP rs11928865 has been described as a risk factor for ARHL. In our sample, it was found a significant difference between genotypes for HL at 8000 Hz, however this results were lost when a multivariate logistic regression controlled by age was conducted. T allele in *GRM7* is the most common in our sample of individuals with presbycusis. Genotypes A/T and T/T present respectively, 1.93 and 2.08 times higher hearing loss risk compared to A/A genotype, even though it is not statistically significant.

Luo *et al* (2013) did a cluster analysis to group audiogram patterns and only found an association between *GRM7* SNP rs11928865 and subjects with "sloping "and "abrupt loss" audiogram phenotype patterns in which anatomical function pathology was observed in the hair cells and in the spiral ganglion nerve cells [29]. Similarly, studies conducted in European descendent (Non-Finnish and white American) population have described an association between *GRM7* SNP rs11928865 and ARHL [28,30]. The major difference between our study and the previous is sample size, which might justify the differences is statistical significance. Friedman *et al* (2009) have used human temporal bone specimens to localize mGluR7, which was detect by immunohistochemistry in the inner and outer hair cells and Hensens' cells of the organ of Corti, in spiral ligament and the spiral ganglion neurons. From this study they hypothesize that an unidentified causative allele of *GRM7* alters synaptic autoregulation of glutamate in the synaptic cleft of the sensory cells of the inner hair cells and the auditory neurons, leading to higher levels of glutamate, with subsequent neurotoxic effects and sensory cell death, thus leading to ARHL [28].

Oxidative stress is thought to contribute to the age process and consequently to ARHL. *NAT2* is responsible for the detoxification of exogenic substrates and are vital to the oxidative balance. *NAT2* is an isoenzyme highly polymorphic. Some of these polymorphisms may result in slow or rapid acetylators, having been both linked to diseases, such as cancer [31]. In our sample, rapid acetylators have the lowest hearing loss and slow acetylators have the highest hearing loss, although no statistically significant differences were found. Nevertheless, it was observed a difference in the haplotypes at 250 Hz, namely between NAT2*5B/NAT2*5B and NAT2*4/NAT2*6A, being the first the better listener and the 6A the worst listener. No other differences were found associating HL and *NAT2* haplotypes. NAT2*6 and NAT2*5 lead to decreased protein expression and NAT2*5 leads to the greatest reduction in acetylation [31,77].

Previous studies have identified NAT2*6A as a susceptibility genotype for ARHL, however these studies only focused on genotyping by real-time PCR 4 [31] or 5 [78] of the slow acetylator causing genotypes, therefore the differences in our results may be due to the higher number of SNPs evaluated, allowing to distinguish more haplotypes.

The haplogroups of mtDNA are a result of the evolution and migration of the modern human, presenting continental specific distribution, thus they are a product of adaptation to the environment. The ancestral-susceptibility model supports that some mtDNA haplogroups may confer certain disease susceptibility, as well as the penetrance of the clinical phenotype, with the change of environment and lifestyle in modern time. As the mtDNA haplogroups are characterized by distinct sets of variants, it is possible that certain mtDNA haplogroups can affect respiratory chain functions and may further perturb the crosstalk signalling [79,80]. In our sample, it was possible to identify 15 haplogroups (H, HV, I, J K, L, M, N, R, T, U, V, W, X and Y), from which it is possible to distinguish 11 European haplogroups (H, HV, I, J, K, R, T, U, V, W, and X), the typically African L haplogroup and 3 Asian haplogroups (M, N and Y) [80]. This high mixture of haplogroups in the Portuguese population might result from the historical expansionism and colonialism that might of allowed the mixture of people from different genetic backgrounds.

The haplogroups with the highest HL is haplogroup X and with the lowest HL is haplogroup N. The most common haplogroup in our sample is haplogroup H, with a 34.12±15.30 mean HL, which is agreement with the observed in the European population [80]. In our sample, no haplogroup was identified as susceptible to ARHL. This is not in concordance with the literature, as Manwaring *et al* (2007) found an association between ARHL and haplogroups U and K [81] and Mostafa *et al* (2014) described a relation between haplogroup U and ARHL in a Egyptian and Italian cohort [82].

These results suggest that the impact of other variables, such as environment, lifestyle and stochastic element, may have prevailed over the genetic factor, weakening the relation between *GRM7*, *NAT2*, mtDNA and ARHL. It also increases the relevance of identifying the various determinants of ARHL, leading to more targeted prevention programs.

Nowadays it is possible to assay thousands of genetic variants in many individuals with high throughput techniques, accelerating the identification of susceptibility genes. The existence of reproducible quantitative traits of the disease is fundamental to these advances and ARHL benefits from this, has it can be evaluated by pure-tone and speech thresholds and supra-threshold hearing measures [30].

The use of only threshold data for a genetic analysis of ARHL can suffer a reduction in power due to genetic heterogeneity [30]. Therefore, the use of only one test for the auditory assessment of the ARHL cohort is one of the limitations of this study, as different tests could have evaluated different aspects of the auditory system. Moreover, comparison of prevalence estimates from different studies is very difficult even when applying the same definition of HL, due to the differences in demographic characteristics across cohorts, especially age.

The MD and VM patients sample underwent a complete neuro-otological evaluation, clinical history questionnaire, cytokine production quantification and gene expression array, in an attempt to find response differences between these diseases.

Our results have found that basal levels of IL-1RA are increased in patients with VM comparing to healthy controls. MD patients with low levels of cytokines have lower production of IL-1RA and MD patients with high levels of cytokines have higher production of all cytokines, when compared to VM and healthy controls. Therefore, these observations allow us to distinguish VM from MD.

The genes in the IL-1 complex code for three cytokines: IL-1α, IL-1β and the IL-1RA. The severity of a given infection is influenced by the balance between the levels of IL-1β and that of IL-1RA. In healthy individuals, IL-1RA is readily detectable in plasma and IL-1β levels are usually undetectable [83]. Interestingly, IL-1RA levels were found to be much higher elevated in patients with MD and VM regardless of the levels of IL-1β. Since TLR10 is an anti-inflammatory pattern-recognition receptor able to up-regulate IL-1RA [84], and the TLR10 allelic variant rs11096955 has been associated with faster hearing loss progression in bilateral MD [85] , the activation of TLR10 could explain the increased levels

of IL-1RA found in patients with MD. Moreover, individuals bearing the TLR10 variant rs11096957 displayed increased levels of IL-1β, TNF-α and IL-6 upon ligation of TLR2 [84]. Likewise, it has been described that there is an increase of IL-1RA in patients during episodes of migraine with and without aura [86].

There is a much higher prevalence of allergy amongst MD patients than in healthy people. As there is a high overlap of symptoms between MD and VM, we investigated if the response to allergens (*Aspergillus* and *Penicillium*) might allow to distinguish these pathologies.

Our results demonstrated that allergenic extracts from *Aspergillus* and *Penicillium* induce a proinflammatory immune response involving TNF-α and IL-6. This response is also more intense in VM than MD. Previous studies have established a relationship between mold extracts and proinflammatory cytokines in autoimmune inner ear disease (AIED) [87], demonstrating that when PBMC from patients with AIED were exposed to mold, it resulted in an up-regulation of IL-1β mRNA expression, and enhanced IL-6 and IL-1β secretion, suggesting that mold acts as a PAMP in a subset of these patients. Since most of our patients did not have a comorbid autoimmune disease, the elevation TNF-α and IL-6 observed in patients with MD after exposure of PBMC to *Penicillium* and *Aspergilus* cannot be explained by another autoimmune condition. Furthermore, it is well established that mold can cause respiratory symptoms and may worsen the course of bronchial asthma, which could lead to increase secretion of TNF-α, IL-6, IFN-γ or IL-1β [88,89].

We found the CD28 signaling in T Helper cells and PI3K signaling in B Lymphocytes canonical pathways were inhibited in non-stimulated PBMC of MD patients with ↑-levels of cytokines when compared to PBMC from VM patients. CD28 is a co-receptor of TCR-CD3 complex and provides a co-stimulatory signal for T-cell activation. Naïve T-cell priming depends on the interaction between CD28, CD80 and CD86, inducing subsequently the production of IL-2 and clonal expansion for effective immune response. In naïve T-cells, CD28 co-stimulation increases cell cycle entry and facilitates the activation of anti-apoptotic program. Also, CD28 co-stimulation enhances the acquisition of T-helper cell-1 and 2 phenotypes [90,91]. Phosphoinositide 3-kinases (PI3K) regulate numerous biological processes, such as cell growth, differentiation, survival, proliferation, migration and metabolism. B-cell development is facilitated by class I and III PI3K, through its defined stages, resulting in at least three distinct lineages of mature B-lymphocytes. In B-cells, PI3K is activated within seconds of antigen-receptor triggering [92]. Also, PI3K has been associated to the promotion of hair cell survival via its downstream targets [93]

On the other hand, iCOS-iCOSL signaling in T-helper cells and dendritic cell maturation canonical pathways are activated in non-stimulated PBMC of MD patients with ↑-levels of cytokines when compared to PBMC from VM patients. Icos is stimulated by T-cell activation and is very low expressed on naïve T-cells. ICos-mediated signal contributes mainly to the regulation of activated T-cells and effector T-cell functions. The potency of ICos is enhanced following ICos ligation by ICosL (inducible T-cell co-stimulator ligand), which increases the production of cytokines. ICos-mediated signaling mainly generates Th1 and Th2 responses [94,95]. Dendritic cell maturation leads to the upregulation of MHC class II and co-stimulatory molecules at the cell surface, CCR7-dependent migration to lymph nodes and promotes the release of cytokines that promote the differentiation of naïve T-cells, as well as other immune cells. Depending on the nature of the stimuli they sense, dendritic cells can produce distinct cytokines, such as IL-12, IL-15, IL-6 and TNF, triggering the differentiation of different types of effector T cells, allowing the adaptation of T-cell polarization to the specific nature of the threat [96].

Taking into account the role of each of the pathways, differently expressed in non-stimulated PBMC of MD patients with ↑-levels of cytokines and PBMC from VM patients, in T-cell differentiation, activation and regulation and B-cell development, we can predict that T-cell activation and B-cell development is reduced in MD when compared to VM. Considering the role of PI3K in hair cell survival,

after gentamicin conditioning [93] and the role of CD28 in the anti-apoptotic program, as they are down regulated in MD, it is possible that these pathways may lead to a higher cell death in MD and have a role in SNHL in MD. These results suggest that CD28 signaling in T-cells and PI3K signalling in B-cells pathways might have a natural underexpression and, iCOS-iCOSL signaling in T-helper cells and dendritic cell maturation pathways an overexpression in MD patients with ↑-level of cytokines.

The differences seen in the extracellular levels of IL-6 and TNF-α between VM patients and MD patients with ↑-levels of cytokines (figure 4.7), may be explained by the activated state of the dendritic cell maturation pathway in MD, as maturated dendritic cells can produce TNF and IL-6, which allows a response of T-cells to the stimuli. TNF has also been linked to corticosteroids responsiveness in immune-mediated SNHL [97]. Therefore, this suggests that MD might be more immune mediated than allergic.

Even though there are no statistically significant differences in these pathways, when comparing the gene expression profile of stimulated PBMC from MD against PBMC from VM patients, some of the key genes involved in them are significantly differently expressed (table 4.14 and table 4.15). TCR is activated by stimulation with *Aspergillus*, the pro-inflammatory, IL-2, CD3 and CD28 are activated by stimulation with *Aspergillus* and *Penicillium,* suggesting a T-cell activation and production of IL-2 is provoked by these stimuli. The absence of significance for the pathways may be due to the consideration of all MD patients and not only MD patients with ↑-levels of cytokines for the comparison.

The stimulation with *Aspergillus* and *Penicillium* cause the activation and inhibition of various genes. A comparison of these genes was conducted between MD patients and VM patients.

Rictor is a regulatory component of the mammalian target of rapamycin complex 2 (mTORC2). Smrz *et al* (2014) demonstrated that rictor can function as a negative regulator in signalling events responsible for FcεRI- mediated mast cell degranulation independently of mTOR and by implication mTORC2, regulating critical events such as calcium mobilization and cytoskeletal rearrangement [98]. As this gene is inhibited in MD when compared to VM, when stimulation with *Aspergillus* occurs, it suggests that a more intense allergic event takes place in VM patients, due to the release of histamine through mast cell degranulation[99].

X-box-binding protein 1 (*XBP1*) and Transcription factor 1 (*E2F1*) are seen activated in MD patients when compared to VM and stimulated with *Aspergillus.* XBP1 has a central role in regulating the expression of pro-inflammatory cytokines in macrophages, is essential for the development and survival of dendritic cells, and it is a major regulator of UPR, mediating adaptation to endoplasmic reticulum (ER) stress [100,101]. ER stress is involved in various diseases, namely sensorineural hearing loss. Moreover, XBP1 mRNA splicing is implicated in the induction of autophagy in auditory cells [101]. Transcription factor 1 (E2F1) is necessary to the regulation of the apoptosis process of DC and T cells and also supresses DC maturation [102]. E2F1 also plays a role in *stria vascularis*, spiral ganglion apoptosis and progressive HL, in people with mtDNA A1555G mutation [103]. Therefore, the study of *XBP1* and *E2F1* could be interesting in MD patients, to evaluate if they could have a role in NSHL in these patients.

Prolactin (*PRL*), Hepatocyte growth factor (*HGF*), transcription factor STAT1, interferon type 2 (*IFNA2*), toll-like receptor (TLR) 3 and interferon regulatory factor 3 (*IRF3*) are seen activated in MD patients when compared to VM and stimulated with *Penicilium.*

PRL stimulates the humoral and cellular immune responses, accelerating the breakdown of immune tolerance by promoting the survival, maturation and activation of autoreactive B and T cells, dendritic cells and macrophages. Elevated serum levels of PRL have been reported in several autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus [104].

HGF has a role in DC pathway decision: immune tolerance (triggering IL-10-dependent mechanism) or immunity [105]. On the other hand, HGF signaling has a role in *stria vascularis* development and lack of HGF signaling in the inner ear leads to profound hearing loss in the mouse [106].

STAT1 is a transcription factor predominately involved in the signal transduction by either type I, type II, or type III IFNs. Furthermore, STAT1 regulates Th1 cell-specific cytokine production and controls the proliferation and apoptosis of immune cells. *STAT1* mutations lead to *STAT1* hyperactivation and defective nuclear dephosphorylation, turning the individuals prone to fungal infections. This mutation leads to chronic mucocutaneous candidiasis, associated with exaggerated IFN-γ signaling, which inhibits IL-17 transcription. These patients with increased IFN-γ signaling are also at risk for autoimmune disease. Systemic lupus erythematosus has also been associated to gain of function mutations in *STAT1* [107]. The increase of activity of this gene in MD patients explains the increase of *IFNA2* activity.

TLR3 and TLR4 recognize fungal PAMPs [87]. TLR3 and TLR4 recruit TRIF to trigger activation of TAK1, which activates TANK-binding kinase 1 (TBK1) and IkB kinase ε (IKKe), leading to the activation of IRF3 and induction of IFN-I expression. In the case of TLR4, another adaptor called TRAM is required to recruit TRIF for IRF3 activation. TRIF recruitment can also activate NFkB and MAPK through RIP1 or TAK1. Nuclear factor-kB (NFkB) activation is conserved among all TLR receptors, as well as the induction of pro-inflammatory cytokines such as TNFα and IL-1β. However, the ability to trigger IRF3 activation, and consequently IFN-I expression, is restricted to TLR4 and the nucleic acid sensing TLRs (TLR3, 7, 8, and 9) [96].

Prostaglandin E receptor subtypes EP4 (*PTGER4*) and *Trim24* are seen inhibited in MD patients when compared to VM and stimulated with *Penicillium*.

Prostaglandin E1 has been used as a corticosteroid treatment for SNHL. PTGER4 mediates the physiological actions of prostaglandin E1 and E2. PTGER4 is coupled to G-protein stimulation, and mediates increases in cyclic adenosine monophosphate (cAMP) that activate protein kinase A (PKA). A study, demonstrates the involvement of PTGER4 in the regulation if VEGF synthesis in the inner ear (spiral ganglion neurons, the cochlear sensory epithelium, spiral ganglion, spiral ligament, and *stria vascularis*). PTGER4-mediated VEGF synthesis in the cochlea may be associated to protection against noise [108].

Trim24 proteins are involved in gene regulation and cytokine signaling. Deletions in *Trim24* do not lead to explicit autoimmune phenotype, however it is essential for Th2 cell-mediated airway allergy and Th2-dependent expulsion of intestinal helminthes [109].

The state of the above genes in MD after stimulation by *Penicillium* suggests an autoimmune involvement and a higher reaction to mold. Contrastingly, the inhibition of *Trim24* may suggest a higher allergic response in VM. Considering the role of *HGF* in HL and *PTGER* in the protection against HL, *HGF* activation and *PTGER4* inhibition may be indicative of a link to SNHL in MD patients.

Interferon-γ (*IFNG*), Interferon-α, tumor necrosis factor (*TNF*), *IL-2, NFkB, myc, TLR4, IL-1A* and *HIF1A* are seen activated in MD patients when compared to VM and stimulated with *Aspergillus* or *Penicillium*. On the other hand growth factor independent factor 1 (*GFI1*), *CD3* and *CD28* are seen inhibited under the same conditions.

NFkB is essential to the regulation of apoptosis by controlling the expression of various anti-apoptotic factors [90]. Two potential regulatory variants in the *NFKB1* gene (rs3774937 and rs4648011) were associated with a faster hearing loss progression in patients with unilateral SNHL. Steroids are potent blockers of *NFkB* pathway, which may explain the observed response to systemic steroids in patients with sudden SNHL or MD [110]. It has been shown that TNFα induces a stronger activation of NFkB in the presence of IFN-γ [111]. Therefore, the *TNF* activated state explains the increase in extracellular TNFα levels, which leads to a consequent activation of *NFkB*, after stimulation with mold.

Myc is dominant over the hypoxia-inducible factor 1α (*HIF1α*) in regulating differentiation of naïve T cells into T_{EFF} cells. HIF1α is vital in the metabolic switch to aerobic glycolysis that influences the balance of Th17/T_{REG} in favour of Th17 cells. T cells with constitutively elevated HIF1α display

sustained increase of aerobic glycolysis and constitutively maintain effector function, reviling the essential role of HIF1α [112].

GFI1A has an important role in controlling natural T_{REG} development by regulating IL-2 production from convectional T-cells [113]. Wallis *et al* (2003) found that knock-out of GFI1 in mice leads to defects that are consistent with inner ear anomalies, as they are ataxic, deaf and have head tilting behaviour, being both the vestibular and cochlear hair cells affected. Gfi1 is expressed in the developing nervous system, is necessary for inner ear hair cell differentiation and its loss causes programmed cell death[114].

The state of these genes in the response to both molds reveal the involvement of an higher immune response and support the higher SNHL prevalence in MD patients than in VM patients.

Various pathways are altered in response to *Aspergillus* and *Penicillium* in VM patients comparing to healthy controls.

IL-8 is a key chemoattractant and activator of neutrophils. The most prominent producer of IL-8 in vitro is the monocyte population of PBMC. There are numerous non-specific cytokines, ligands and metabolic stimulants that induce *IL-8* gene expression in monocytes and PBMC, however it has been described that in sites of TNFα accumulation there is a high production of IL-8 [115]. The IL-8 signaling pathway is seen inhibited when stimulated with *Aspergilus* and activated when stimulated with *Penicillium.* This may be explained by the higher production of TNFα in PBMC stimulated with *Penicillium* (figure 4.6).

TLR are characterized pattern recognition receptors (PRRs), able to detect a variety of pathogen-associated molecular patterns (PAMPs), such as lipids, proteins, lipoproteins, and nucleic acids. TLR activation can induce inflammatory cytokines (TNF-α, IL-6), co-stimulatory molecules, type I and type II and interferon and chemokines. All TLRs except TLR3 utilize MyD88 for the production of inflammatory cytokines or type I IFNs (TLR7, TLR8, and TLR9). TLR activates NFkB and MAPKs for regulation of pro- and anti-inflammatory cytokine production [116,117]. Toll-like Receptor Signaling and NFkB signalling pathways are inhibited in VM patients compared to healthy controls, however observing the level of extracellular cytokines obtained in our results it is possible to conclude that these pathways have been active. A possible explanation for this is that the response of VM patients was more intense and all the mRNA was converted into protein before the harvest of the cells and RNA extraction.

Apoptosis plays a role in the shut-down of cellular as well as humoral immune responses. In the case of acute immune response, foreign antigen-specific T and B cells can expand several hundred-fold in numbers, which differenciate into effector cells, to overcome the pathogens by killing them, either directly or indirectly through cytokine-mediated activation of cells of the innate immune system. After the defeat of the pathogen, effector cells undergo programmed death, as they are no longer needed, restoring normal cellularity. Also, apoptosis of activated lymphocytes minimizes the collateral damage to healthy tissues that can be caused by the immune effector molecules (e.g. inflammatory cytokines, immune complexes, perforin-granzymes) [118]. The inhibited state of the Death Receptor Signaling pathway in VM patients suggest that VM patients may take longer to activate t-cell apoptosis after stimulation with mold.

6. Conclusion and further directions

This study has focused on inner ear disease and as allowed us to draw the following conclusions:

1) Inner ear disorders are very complex and have many possible causes as well as comorbidities;
2) Our results show that the Portuguese ARHL population has various types of comorbidities, such as cholesterol and diabetes.
3) Regarding the genetic factors evaluated, *GRM7* SNP rs11928865 A/A genotype and NAT2*4/NAT2*6A have the lowest HL.
4) For our sample, mtDNA haplogroup showed no effect on HL.
5) The combined results on the study of ARHL suggest that in our population environmental factors may have a higher input into ARHL than the genetic factors evaluated.
6) VM patients can be distinguished from MD patients and healthy controls according to their IL-1RA levels without stimulation.
7) *Aspergillus* and *Penicillium* provoke an increase in IL-6 and TNF-α production in VM and MD, which although distinguishable from healthy controls, the levels of stimulation among the diseases is very similar.
8) The microarray results unveiled the involvement of many genes and pathways in MD and VM, which suggest a higher allergic response in VM.
9) The microarray data is supportive of the findings in the extracellular cytokine measurements.

ARHL should be perceived as a disease of preventable nature as well as treatable. Therefore, further genetic screening should be conducted, for identification of functional variants that will allow impact assessment of genotype on phenotypic outcomes of interest. Also proteomics and microarrays may be used to detect predictors of disease susceptibility. This combined information can reveal new mechanisms involved in ARHL, hinting towards pharmaceutical targets and screening kits.

The next steps, in the study of VM and MD, should be conducting longitudinal experiments in the same patients, to assess if the increase in the cytokines is persistent or fluctuating over time; try to further investigate the downstream effects of IL-6 and TNF-α to define candidate targets for therapy and to define additional cytokines in separated immune cell populations to figure out which cell types are driving the response to molds.

The recognition of different subgroups of patients in MD is the first step to improve the selection of patients for genetic and immunological studies, therefore increasing the number of patients with MD type 4 in our sample would be advantageous in studies to further differentiate MD from VM, has it is the MD type with most overlapping symptoms.

7. References

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8. Annexes

A. WHO standardized hearing loss classification

Table 8.1 – WHO standardized hearing loss classification

WHO Classification	Hearing loss (dB)	Hearing Stage
Normal	<26	0
Mild	26-40	1
Moderate	41-60	2
Severe	61-80	3
Profound	>80	4

B. Diagnostic criteria for VM

A. At least 5 episodes fulfilling criteria C and D.
B. A current or past history of migraine with(out) aura.
C. Vestibular symptoms of moderate or severe intensity, lasting between 5 minutes and 72 hours.
D. At least 50% of episodes associated with at least 1 of the following 3 migrainous features:
   i. Headache with at least 2 of the following 4 characteristics: unilateral headache; pulsating quality; moderate to severe intensity; or aggravation by routine physical activity
   ii. Photophobia and phonophobia
   iii. Visual aura
E. Not better accounted for by another ICHD-3β diagnosis or by another vestibular disorder.

C. PCR protocols

Table 8.2 - mtDNA PCR protocol.

	Temperature (ºC)	Time	Number of cycles
Initial denaturation	95	3’	1
Denaturation	95	15’’	35
Annealing	60	15’’	
Extension	72	15’’	
Final extension	72	1’	1

Table 8.3 - NAT2 PCR protocol

	Temperature (ºC)	Time	Number of cycles
Initial denaturation	95	3’	1
Denaturation	95	15’’	35
Annealing	61.6	15’’	
Extension	72	1’’	
Final extension	72	1’	1

Table 8.4 - GRM7 real-time PCR protocol

	Temperature (ºC)	Time	Number of cycles
Pre-PCR	60	30''	1
	50	2'	1
Initial denaturation	95	10'	1
Denaturation	95	15''	40
Annealing	60	30''	40
Extension	72	30''	40
Post-PCR	60	30''	1

Table 8.5 - qPCR protocol

	Temperature (ºC)	Time	Number of cycles
Pre-PCR	95	20''	1
Denaturation	95	1''	40
Annealing/Extension	60	20''	40

D. Demographic Results

Table 8.6 – Influence of demographics and clinical history on HL based on the WHO classification.

	Normal (≤25 dB)	HL (>25 dB)	p-value
Sex			
Feminine	77	151 (66.2%)	0.716
Masculine	36	78 (68.4%)	
Age			
mean±SD	68.65±7.96	78.37±8.53	**<0.001***
Cholesterol			
Yes	69	91 (56.9%)	**0.009***
No	34	90 (72.6%)	
Hypertension			
Yes	54	127 (70.2%)	**0.022***
No	48	62 (56.4%)	
Tinnitus			
Yes	74	119 (61.7%)	0.520
No	33	65 (66.3%)	
Diabetes			
Yes	21	58 (73.4%)	0.124
No	73	127 (63.5%)	
Smoking			
Yes	30	43 (58.9%)	0.327
No	77	147 (65.6%)	
Cardiovascular Disease			
Yes	17	69 (80.2%)	**0.003***
No	77	126 (62.1%)	
Noise Exposure			
Yes	31	70 (69.3%)	0.162
No	77	119 (60.7%)	
Ototoxic Medication			
Yes	23	29 (55.8%)	0.520
No	68	108 (61.4%)	

E. *NAT2* haplotype

Table 8.7 - Distribution of individuals according to *NAT2* haplotype and associated mean HL±SD

Genotype	Frequency (%)	HL±SD (dB)
4/5U	70 (21.0)	37.02±16.19
4/5B	60 (18.0)	34.92±17.01
5B/5B	53 (15.9)	35.66±16.64
4/6A	49 (14.7)	30.46±13.56
6A/6A	24 (7.2)	31.51±13.87
4/4	19 (5.7)	31.91±16.46
4/5A	7 (2.1)	37.14±21.19
5A/5B	6 (1.8)	26.67±7.97
4/5V	3 (0.9)	32.08±15.02
4/7B	3 (0.9)	32.92±10.10
5B/5C	3 (0.9)	30.00±16.54
6J/13A	3 (0.9)	31.67±11.34
12A/5B	2 (0.6)	44.38±7.96
4/12C	2 (0.6)	27.50±0.00
4/5G	2 (0.6)	34.38±29.17
5B/12A	2 (0.6)	29.38±4.42
5B/5D	2 (0.6)	20.63±11.49
11A/5B	1 (0.3)	49.75±NA
12A/12C	1 (0.3)	21.25±NA
12B/12B	1 (0.3)	35.00±NA
13A/13A	1 (0.3)	38.35±NA
13A/14B	1 (0.3)	60.00±NA
4/12A	1 (0.3)	23.75±NA
4/12B	1 (0.3)	43.75±NA
4/13A	1 (0.3)	16.25±NA
4/14B	1 (0.3)	46.25±NA
4/5D	1 (0.3)	60.00±NA
4/5J	1 (0.3)	23.75±NA
4/5R	1 (0.3)	47.50±NA
5A/11A	1 (0.3)	31.25±NA
5B/13A	1 (0.3)	28.75±NA
5B/6A	1 (0.3)	52.50±NA
5D/5G	1 (0.3)	7.50±NA
5R/12A	1 (0.3)	27.50±NA
5U/6B	1 (0.3)	61.25±NA
6A/13A	1 (0.3)	15.63±6.19
6A/6B	1 (0.3)	18.75±NA
6J/7B	1 (0.3)	31.25±NA
6N/6N	1 (0.3)	17.50±NA

F. mtDNA haplotypes

Table 8.8 - Distribution of individuals according to mtDNA haplogroup and associated mean HL±SD.

Haplogroup	N (%)	Mean HL± SD (dB)
H	172 (51.19)	34.12±15.30
U	35 (10.42)	31.86±14.77
HV	24 (7.14)	35.05±14.97
T	23 (6.85)	38.42±19.13
L	19 (5.65)	28.62±11.92
J	16 (4.76)	33.91±14.84
X	11 (3.27)	43.30±16.04
K	9 (2.68)	36.11±20.89
V	9 (2.68)	33.75±22.83
R	5 (1.49)	42.00±18.15
I	4 (1.19)	41.56±28.82
W	4 (1.19)	40.63±10.92
N	3 (0.89)	19.58±4.39
M	1 (0.30)	23.75±NA
Y	1 (0.30)	43.75±NA